Functional Analysis of Dehydratase Domains of a Polyunsaturated fatty acid Synthase from *Thraustochytrium* sp. by Mutagenesis

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By

Kaiwen Sun

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ABSTRACT

Very long chain polyunsaturated fatty acids (VLCPUFAs), such as docosahexaenoic acid (DHA, 22:6-4,7,10,13,16,19) and eicosapentaenoic acid (EPA, 20:5-5,8,11,14,17), are essential for humans and animals since they are parts of the cell membrane and are involved in mediating various physiological processes. *Thraustochytrium* sp. ATCC 26185 is a marine protist that can produce large amounts of VLCPUFAs such as DHA and DPA (docosapentaenoic acid, 22:5-4,7,10,13,16) for human food and animal feed. Biosynthesis of these fatty acids in *Thraustochytrium* is catalyzed by a polyunsaturated fatty acid (PUFA) synthase that comprises three subunits, each with multiple catalytic domains. Three dehydratases (DH) domains in the PUFA synthase are believed to be responsible for coordinately introducing multiple double bonds in VLCPUFAs; however, the exact function of these domains remains to be determined. In this research, two DH domains (DH1 and DH2) in subunit-C of the PUFA synthase that have sequence similarity to *E. coli* FabA were functionally analyzed by site-directed mutagenesis and domain deletion analyses. Site-directed mutagenesis analysis showed that mutation of a histidine residue at calalytic site into alanine in DH1 of the PUFA synthase resulted in the complete loss of activity in the biosynthesis of all VLCPUFAs. Mutation of catalytic residue histidine into alanine in DH2 resulted in the production of a small amount of DPA, but not DHA. In addition, the deletion of DH1 domain also led to the complete loss of function while deletion of DH2 domain resulted in the production of only a very small amount of DPA. These results indicate that two FabA-like domains of the PUFA synthase possess distinct functions. DH1 domain, but not DH2 domain, is essential for the biosynthesis of VLCPUFAs, and DH2 domain is required for the biosynthesis of DHA. The PUFA synthase must have both for the efficient production of VLCPUFAs. Next, expression and purification of the PUFA synthase were attempted for future structure analysis. Partial purification of these subunits was accomplished using a His-tagged protein purification system and verified with western blot analysis. Successful purification of the subunit of the PUFA synthase expressed in *E. coli* would be an important step forwards for studying the structure and activity of each subunits of this enzyme and offer strategies for elucidating the molecular mechanism for the biosynthesis of VLCPUFAs.

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LIST OF ABBREVIATIONS

ACP	Acyl carrier protein
ALA	α-linolenic acid
ARA	Arachidonic acid
AT	Acyltransferase
BLAST	Basic local alignment search tool
CLF	Chain length factor
CoA	Coenzyme A
DAB	3,3'-diaminodbenzidine
DH	Dehydratase
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DPA	Docosapentaenoic acid
E. coli	*Escherichia coli*
EDTA	[[2-(Bis-carboxymethyl-amino)-ethyl]-carboxymethyl-amino] acetic acid
EPA	Eicosapentaenoic acid
ER	Enoyl reductase
FabA	3-hydroxyacyl-ACP dehydratase
FabZ	3-hydroxyacyl-ACP dehydratase
FAME	Fatty acid methyl ester
FAS	Fatty acids synthase
GC	Gas chromatography
HP Taq DNA polymerase	High performance Thermus aquaticus polymerase
HRP	Horseradish peroxidase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KR	Ketoacyl reductase
KS	Ketoacyl synthase
LA	Linoleic acid
LB broth	Luria-Bertani broth
LCPUFA	Long chain polyunsaturated fatty acid

LT	leukotrienesLeukotrienes
MAT	Malonyl-acetyl transferase
NCBI	National Center for Biotechnology Information
Ni-NTA	Nickel-Nitrilotriacetic Acid
OD	Optical density
ORF	Open reading frame
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with 0.05% Tween® 20
PCR	Polymerase chain reaction
PG	Prostaglandins
PKS	Polyketide synthase
PMSF	Phenylmethyl sulfonyl fluoride
PPAR	Peroxisome proliferator-activated receptor
PPTase	Phosphopantetheinyl transferase
PUFA	Polyunsaturated fatty acid
PUFA synthase	Polyunsaturated fatty acid synthase
RNA	Ribonucleic acid
rpm	Revolutions per minute
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHR	Spontaneously hypertensive rats
SREBP	Sterol regulatory element binding protein
THA	Tetracosahexaenoic acid
TPA	Tetracosapentaenoic acid
Tris-HCI	Trisaminometano hydrochloride
UV	Ultraviolet
VLCPUFA	Very long chain polyunsaturated fatty acid

1. INTRODUCTION

1.1 Overview

Very long chain polyunsaturated fatty acids (VLCPUFAs) such as docosahexaenoic acid (DHA, 22:6-4,7,10,13,16,19) and eicosapentaenoic acid (EPA, 20:5-5,8,11,14,17) are essential for human health. Supplementation of VLCPUFAs has shown that these fatty acids can improve the retinal and neural development, and provide protection against inflammation and cardiovascular diseases (Bazan et al., 2010; Janssen & Kiliaan, 2014; McNamara et al., 2013). In nature, the biosynthesis of VLCPUFAs goes through either an aerobic pathway or an anaerobic pathway. The anaerobic pathway is catalyzed by a PUFA synthase that mainly occurs in microorganisms. *Thraustochytrium* sp. ATCC 26185 is a unicellular marine protist that can produce a high level of VLCPUFAs using a PUFA synthase. The PUFA synthase comprises three subunits (subunit-A, -B and -C), each subunit with multiple catalytic domains. Three dehydratases (DH) domains are identified in the PUFA synthase. One is located in subunit-A (DH-A), and the other two DH domains (DH1 and DH2) are adjacently located in subunit-C. These three DH domains in the PUFA synthase are assumed for coordinately introducing multiple double bonds in VLCPUFAs; however, the exact function of these domains remains to be determined. The aim of this study was to functionally analyze two FabA-like DH domains (DH1 and DH2) in subunit-C of the PUFA synthase by site-directed mutagenesis and domain deletion analyses.

1.2 Hypothesis

Dehydratase (DH) domains of the PUFA synthase in *Thraustochytrium* sp. ATCC 26185 catalyze the dehydration reaction, an essential process for intricately positioning multiple double bonds in VLCPUFAs.

1.3 Objective

The objectives of this research were as follows.

1. Sequence analysis of the DH domains in the PUFA synthase from *Thraustochytrium* sp. ATCC 26185.

2. Mutagenesis of DH1 and DH2 domains in subunit-C of the PUFA synthase from *Thraustochytrium* using *in vitro* site-directed mutagenesis and domain deletion.
3. Functionally analyze the mutated domains by the heterologous expression of them along with the other domains of the PUFA synthase in *E. coli.*
4. Expression and purification of the PUFA synthase for future structural analysis.

2. LITERATURE REVIEW

2.1 Very long chain polyunsaturated fatty acids (VLCPUFAs)

Very long chain polyunsaturated fatty acids (VLCPUFAs) refer to fatty acids with the chain length longer than 18 carbons and with two or more double bonds. According to the position of double bonds, these fatty acids can be divided into two groups, ω-3 and ω-6 VLCPUFAs (Figure 2.1). Omega-3 VLCPUFAs have the first double bond located at the third carbon away from the methyl (CH_3) end, while omega-6 VLCPUFAs have the first double bond at the sixth carbon away from the methyl end.

2.1.1 Omega-3 VLCPUFAs

Omega-3 VLCPUFAs are vital for human health and important in human nutrition. Two important ω-3 VLCPUFAs for dietary supplementation are eicosapentaenoic acid (EPA, 20:5-5,8,11,14,17) and docosahexaenoic acid (DHA, 22:6-4,7,10,13,16,19). These ω-3 fatty acids are naturally found in oceanic fish and marine microorganisms. However, the oceanic fish source is declining due to overfishing and environmental issues (Tocher et al., 2019). Therefore, marine microorganisms have become increasingly important to supply these ω-3 VLCPUFAs, as they are able to *de novo* synthesize VLCPUFAs and can produce a large quantity of VLCPUFAs in a short time period under culture conditions.

2.1.2 Omega-6 VLCPUFAs

One of the nutritionally important ω-6 VLCPUFAs is arachidonic acid (ARA, 20:4-5,8,11,14), which is mainly found in meat, poultry, egg and dairy foods (Li et al., 1998). In humans, ARA is the most abundant ω-6 VLCPUFA with multiple physiological activities. It is present in the phospholipids of the cell membrane and released in response to cell injury through the action of phospholipase (Korotkova & Lundberg, 2014). After release from membrane lipids, ARA can be metabolized to pro-inflammatory eicosanoids, bioactive compounds that regulate many physiological processes (Sprecher, 2002).

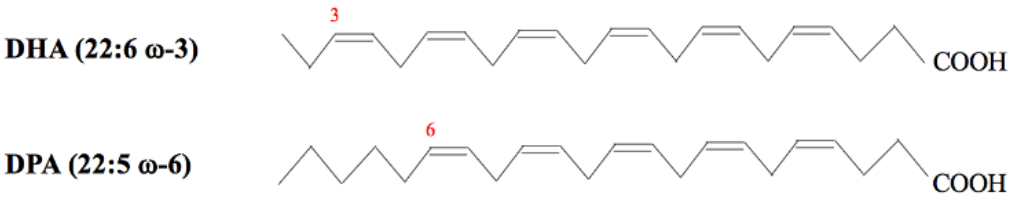


Figure 2.1 Two types of VLCPUFAs, such as DHA (ω-3) and DPA (ω-6).

2.2 VLCPUFAs and human health

VLCPUFAs are essential components in human cells where they can be incorporated into the phospholipid bilayer of cell membranes, thereby affecting membrane fluidity and membrane protein activity (Brash, 2001). Among the VLCPUFAs, ω-3 VLCPUFAs have drawn particular attention due to their importance in the health and wellbeing of humans and animals. Omega-3 VLCPUFAs are widely regarded as cardioprotective; therefore, supplementation of ω-3 VLCPUFAs is recommended to prevent the occurrence of cardiovascular diseases. High blood pressure is a risk factor for various cardiovascular diseases, such as myocardial infraction, stroke and heart failure (Janssen and Kiliaan, 2014). A recent study suggests that long-term supplementation of krill oil, which is rich in EPA and DHA, could significantly decrease blood pressure in spontaneously hypertensive rats (SHRs) (Zhou et al., 2017). Both ω-3 and ω-6 VLCPUFAs are precursors for the biosynthesis of signaling molecules, eicosanoids and docosanoids. Eicosanoids are bioactive molecules that are involved in a variety of physiological activities, such as inflammation and platelet aggregation (Schmitz & Ecker, 2008; Serhan, 2005). For instance, ARA is the precursor of pro-inflammatory eicosanoids 4 series leukotrienes (LT_4) and 2 series prostaglandins (PG_2), while EPA is the precursor of anti-inflammatory mediators 3 series prostaglandins (PG_3) and 5 series leukotrienes (LT_5) (Endo & Arita, 2016). DHA can be metabolized to anti-inflammatory docosanoids resolvin E1 and neuroprotectin D1, which can prevent the conversion of ARA into pro-inflammatory eicosanoids (Gabbs et al., 2015). VLCPUFAs can also directly regulate gene expression by interacting with transcriptional factors, including nuclear receptor peroxisome proliferator-activated receptor (PPAR), retinoid X receptor (RXR) and sterol regulatory element-binding protein (SREBP) (De Urquiza et al., 2000; Jump et al., 1996). SREBPs are important nuclear transcription factors initially located on endoplasmic

reticulum to maintain lipid metabolism homeostasis of cells (Deckelbaum et al., 2006). Dietary intake of VLCPUFAs can affect the activity and expression of SREBPs. Brains are rich in VLCPUFAs. The concentration of VLCPUFAs in brains is generally believed to decrease with aging, which is accompanied by declined neuronal survival and loss of grey and white matter volume. DHA can act as a neurotrophic factor for the increased activation of synaptic proteins, which enhances synaptic plasticity and cognition function (Masliah et al., 2006; Moretti et al., 2012).

2.3 Biosynthesis of VLCPUFAs

In nature, VLCPUFAs are biosynthesized by two distinct pathways, aerobic and anaerobic pathways. The aerobic pathway involves multiple alternating desaturations and elongation processes, while the anaerobic pathway utilizes a single multifunctional mega-enzyme to synthesize VLCPUFAs from initial acetate precursor (Qiu, 2003).

2.3.1 Aerobic pathway

The aerobic pathway for the biosynthesis of VLCPUFAs involves alternating desaturation and elongation reactions catalyzed by various desaturases and elongases (Figure 2.3.1). Molecular oxygen is required for desaturation process to introduce double bonds. The biosynthesis starts from saturated steric acid (18:0), which is sequentially desaturated by Δ9 and Δ12 desaturases giving linoleic acid (LA 18:2-9,12). LA is then desaturated by Δ15 desaturase to give rise to α-linolenic acid (ALA 18:3-9,12,15). LA and ALA are the precursors for the synthesis of ω-6 and ω-3 VLCPUFAs, respectively. For instance, the desaturation of ALA is catalyzed by Δ6 desaturase, followed by elongation catalyzed by Δ6 elongase, and then desaturation catalyzed by Δ5 desaturase, giving EPA (20:5-5,8,11,14,17). EPA can then be elongated by Δ5 elongase to 22:5-7,10,13,16,19, which is finally desaturated by Δ4 desaturase to DHA (22:6-4, 7, 10, 13, 16, 19) (Abe et al., 2006; Meesapyodsuk & Qiu, 2014; Qiu et al., 2001; Tavares et al., 2011; Ye et al., 2015). It is worth noting that the Δ4 desaturase-dependent process for the synthesis of DHA is only found in eukaryotic microbes. In mammals, the aerobic pathway for the synthesis of DHA goes through two rounds of elongations of EPA to TPA (24:5-9,12,15,18,21), and a Δ6 desaturation to THA (24:6-6,9,12,15,18,21), and then one cycle of β-oxidation for a two carbon

chain shortening, giving rise to DHA (Bazinet & Layé, 2014; Sprecher, 2002; Uauy et al., 2000). It is noted that humans lack Δ12 and Δ15 desaturases for the synthesis of LA and ALA; therefore, these two fatty acids must be supplied by diets in order to complete the synthesis of DHA in humans.

2.3.2 Anaerobic pathway

The anaerobic pathway for the biosynthesis of VLCPUFAs occurs only in microorganisms. It does not require molecular oxygen for desaturations to introduce double bonds. The biosynthesis is catalyzed by a single enzyme called PUFA synthase, which is a polyketide synthase (PKS)-like mega-enzyme with multiple functional domains such as ketoacyl synthase (KS), ketoacyl reductase (KR), dehydratase (DH), enoyl reductase (ER), acyltransferase (AT), malonyl-CoA:ACP transacylase (MAT) and acyl carrier protein (ACP) (Figure 2.3.2). Before starting the biosynthesis, inactive apo-ACP has to be converted to active holo-ACP by attaching phosphopantetheine to ACP domains of a PUFA synthase by phosphopantetheinyl transferase (PPTase). Afterwards, the biosynthesis starts with the condensation of acetyl-ACP and malonyl-ACP by ketoacyl-ACP synthase (KS), producing ketoacyl-ACP, which is then converted to hydroxyacyl-ACP by ketoacyl-ACP reductase (KR). Subsequently, a water molecule is removed from hydroxyacyl-ACP forming unsaturated enoyl-ACP by dehydratase (DH), and this enoyl-ACP is finally reduced to saturated acyl-ACP by enoyl-ACP reductase (ER) in a cycle. The four catalytic reactions are repeated several times until the final product is produced. In some cycles of the reactions, enoyl-ACP reduction steps are skipped, as such double bonds are introduced in VLCPUFAs.

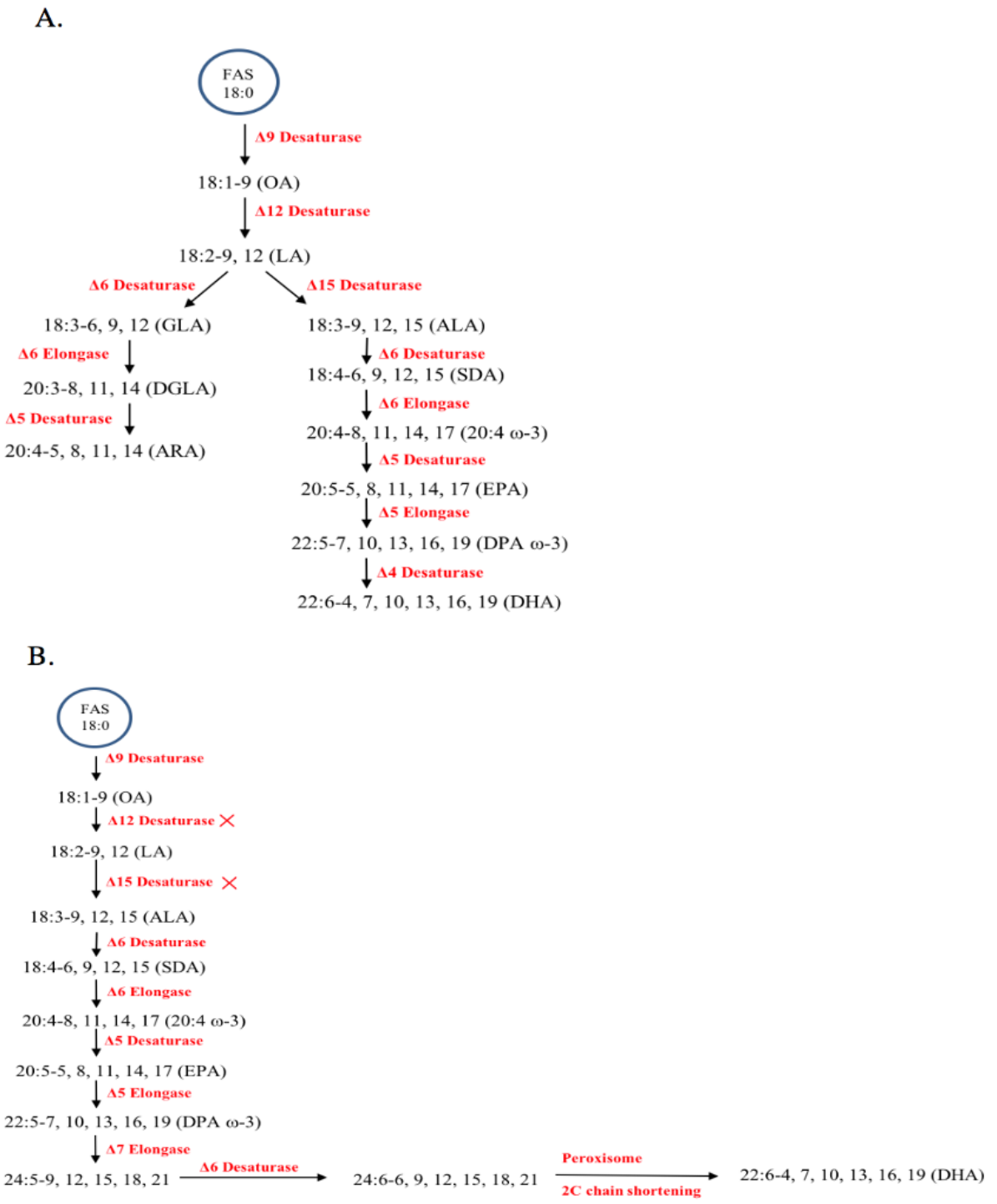


Figure 2.3.1 Aerobic pathway for VLCPUFA biosynthesis. A: The aerobic pathway of DHA in microorganisms; B: The aerobic pathway of DHA in mammals. (Adapted from Qiu, 2003).

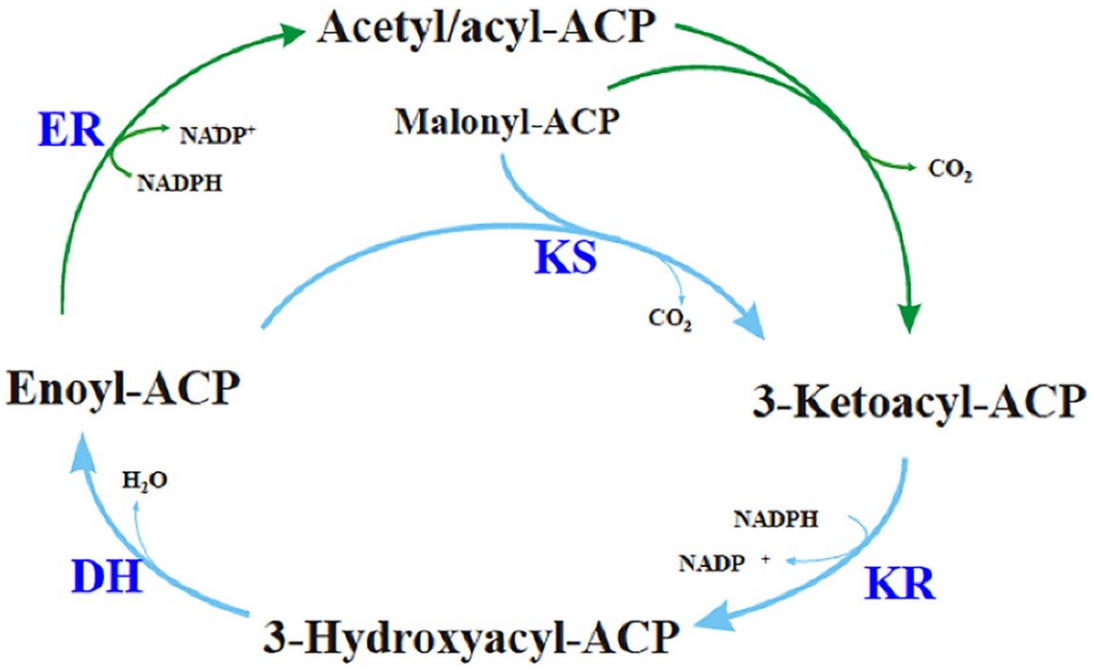


Figure 2.3.2 The anaerobic pathway for VLCPUFA biosynthesis (Meesapyodsuk & Qiu, 2016). ACP, acyl carrier protein; KS, 3-ketoacyl-ACP synthase; KR, 3-ketoacyl-ACP reductase; DH, 3-hydroxyacyl-ACP dehydratase; ER, enoyl-ACP reductase.

2.4 VLCPUFA biosynthesis in *Thraustochytrium*

2.4.1 *Thraustochytrium*

Thraustochytrium sp. ATCC 26185 is a unicellular marine protist that can produce a high level of VLCPUFAs in storage lipids. In nature, many *Thraustochytrium* species can naturally accumulate large amounts of DHA, a nutritionally important VLCPUFA (Lewis et al., 1999). As marine fish are facing issues of over-exploitation and environmental pollution, DHA-producing *Thraustochytrium* becomes a promising source of the fatty acid for dietary supplementation.

2.4.2 DHA biosynthesis pathway in *Thraustochytrium*

Our earlier sequence survey indicates that both aerobic Δ4 desaturase-dependent pathway and anaerobic PUFA synthase pathway co-exist in *Thraustochytrium* sp. ATCC 26185 (Zhao et al., 2016). However, later research indicates that two critical desaturation steps, Δ9 and Δ12 desaturations, are absent from the aerobic pathway despite Δ4, Δ5, Δ6 and ω-3 desaturases and Δ5

and Δ6 elongase are present in this protist. Therefore, it is believed that the anaerobic pathway is solely responsible for the biosynthesis of VLCPUFAs in *Thraustochytrium* catalyzed by a PUFA synthase, a polyketide synthase-like mega-enzyme (Meesapyodsuk and Qiu, 2016). The PUFA synthase from *Thraustochytrium sp.* ATCC 26185 is comprised of three subunits or open reading frame (ORF), each with multiple catalytic domains. Possible functions of some of these domains in the PUFA synthase have been recently analyzed (Xie, 2017, 2018). However, further characterization of these domains is required for elucidating the full molecular mechanism for the biosynthesis of VLCPUFAs catalyzed by the PUFA synthase.

2.5 PUFA synthase

The anaerobic pathway catalyzed by a PUFA synthase was first discovered in marine bacteria, and later also found in protist *Schizochytrium* and *Thraustochytrium* (Metz et al., 2001). Currently, PUFA synthases have been widely identified in prokaryotic bacteria and eukaryotic protists and algae, where they are responsible for the biosynthesis of VLCPUFAs (Figure 2.5). For instance, PUFA synthases are identified for the production of DHA, EPA and ARA in *Moritella marina*, *Photobactierum profundum*, and *Aureispira marina*, respectively (Allen & Bartlett, 2002; Morita et al., 2000; Ujihara et al., 2014). PUFA synthase in these bacteria is encoded by a cluster of *Pfa* genes for four subunits named PfaA, PfaB, PfaC and PfaD; each with one or more catalytic domains. Differences in orientation and order of these domains may lead to the production of different VLCPUFAs. For instance, *Shewanella oneidensis* MR-1 is an EPA producer that is capable of growing under a variety of stress conditions. The *pfa* gene cluster of *S. oneidensis* consists of five open reading frames (*pfa*A-E ORFs) in the genome. *pfa*A is a multifunctional gene encoding five domains: KS, MAT, four repeats of ACP, KR and DH, while *pfa*B encodes a mono-functional protein for possibly AT activity. *pfa*C codes for KS and DH domains and *pfa*D gene encodes a single ER domain. The fifth ORF *pfa*E codes for a phosphopantetheine transferase (PPTase) for activating ACP domains (Lee et al., 2006). In general, amino acid sequences of catalytic domains in both prokaryotic and eukaryotic PUFA synthases are highly conserved; however, the domain number and their organization in subunits of these PUFA synthase can be very different. Typically, there are three subunits in a eukaryotic PUFA synthase, each with multiple catalytic domains. For instance, subunit A of a PUFA synthases from *Thraustochytrium* comprises KS, MAT, eight repeats of ACP, KR and DH domains, subunit B contains KS, CLF, AT and ER domains, and subunit C is composed of two DH and one ER domain

(Meesapyodsuk and Qiu, 2016). In contrast, a prokaryotic PUFA synthase is usually made of four subunits with only one ER domain.

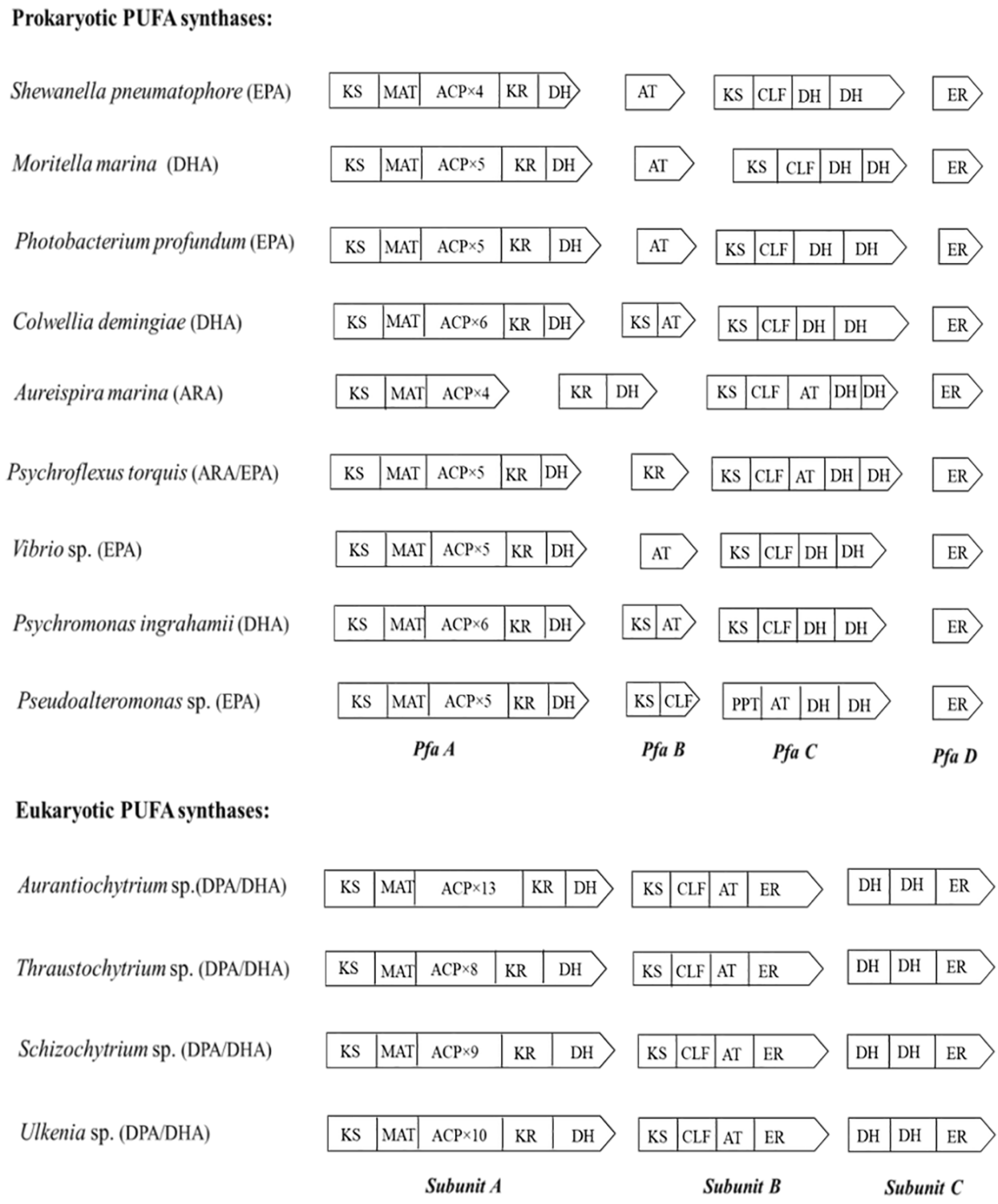


Figure 2.5 Structure of PUFA synthase from microorganisms (Qiu et al., 2020).

2.6 Dehydratase (DH)

Dehydratase (DH) in fatty acid biosynthesis catalyzes the removal of a water molecule from 3-hydroxyacyl-ACP, thereby introducing a double bond into a growing acyl chain. DH domains in a PUFA synthase are assumed to function in the same way. It is noteworthy that a PUFA synthase usually comprises multiple DH domains. According to their sequences similarity, these DH domains can be categorized into two types, PKS-like DH and FabA-like DH domains (Leesong et al., 1996). PKS-like DH domains are assumed to catalyze the dehydration of β-hydroxyacyl-ACP, introducing a 2-trans double bond between α and β–carbon, which will be further reduced by enoyl-ACP reductase (Rouhiainen et al., 2004). FabA-like DH domains are assumed to function as a bi-functional enzyme-like *E. coli* FabA catalyzing the dehydration of 3-hydroxyacyl-ACP to trans-2-enoyl-ACP first, and then isomerization of trans-2-enoyl-ACP to cis-3-enoyl-ACP (Heath & Rock, 1996b), thereby introducing a cis double bond which will be retained in an acyl chain (Hayashi et al., 2019).

2.6.1 Dehydratase (DH) in type II FAS

Type II fatty acid synthase comprises several discrete catalytic enzymes, and it is found in bacteria, eukaryotic mitochondria and plant plastids (Brown et al., 2009; Yoshida et al., 2016). In *E. coli*, there are two dehydratase enzymes FabA and FabZ that catalyze the removal of a water molecule from β-hydroxyacyl-ACP in type II fatty acid synthesis. They share only 28% of amino acid identity. FabA most efficiently dehydrates intermediate acyl-ACP with the chain length of 10 carbons, while FabZ has preference for the dehydration of short and long chain (<8 and> 14 carbons) acyl-ACPs. Structurally, FabA forms asymmetric dimer with active histidine residue from one subunit and active aspartic acid residue from the other subunit located in a tunnel-shaped pocket (Leesong et al., 1996). It has high specificity to substrates with a 10-carbon linear chain, while acyl groups with chain length longer or shorter or branched-chain do not fit into the substrate pocket. FabA is a bi-functional enzyme catalyzing both dehydration of β-hydroxydecanoyl-ACP and isomerization of 2-trans-decenoyl-ACP to 3-cis-decenoyl-ACP (Heath and Rock, 1996). During the fatty acid synthesis, 3-cis-decenoyl-ACP will be condensed with an extender in the following extension cycle, resulting in the cis double bond being retained. Therefore, FabA is essential for the biosynthesis of unsaturated fatty acids in *E. coli*. On the other hand, FabZ catalyzes only the dehydration reaction with a wide range of substrates, such as saturated and

monounsaturated β-hydroxyacyl-ACPs, giving 2-trans-enoyl-ACPs, which will be reduced by enoyl-ACP reductase (FabI) to saturated acyl-ACPs (Lu et al., 2004). Both FabA and FabZ possess a general double "hot-dog" architecture with six strands of antiparallel β-sheets wrapping around a central α-helix. However, the difference can be found in the shape of the substrate channel and active residue at the catalytic site (aspartic acid in FabA is replaced by glutamic acid in FabZ), which might have determined their different substrate specificity and catalytic mode (Kimber et al., 2004).

2.6.2 Dehydratase (DH) domains of PUFA synthase in *Thraustochytrium*

There are two different types of DH domains, PKS-like DH and FabA-like DH in microbial PUFA synthases. PKS-like DH domains might be responsible for introducing trans double bond which will be reduced, while FabA-like DH domains might be responsible for introducing cis-double bonds, which will be retained. Coordination of the two types of DH domains would lead to positioning multiple cis double bonds in VLCPUFAs. The PUFA synthase from *Thraustochytrium* sp. ATCC 26185 comprises three DH domains. One DH domain located in the C-terminal region of subunit-A shares more sequence similarity to DH domains of polyketide synthases; thus, it's likely for introducing a trans double bond, which is subsequently reduced. The other two DH domains that reside adjacently in subunit-C are more similar to *E. coli* dehydratase FabA that probably introduces cis double bonds. Our recent studies showed that two FabA-like DH domains could functionally complement *E. coli* mutants defective in the dehydratase activity of type II fatty acid synthases (Xie et al., 2018). However, functions of the individual FabA-like DH domain of the PUFA synthase from *Thraustochytrium* remains undetermined.

3. EXPERIMENTAL APPROACH

3.1 Materials

3.1.1 *E. coli* strains

E. coli Top10 was purchased from Invitrogen Biotechnology Co. (Grand Island, NY, USA). *E. coli* SHuffle strain was acquired from New England BioLabs (Ipswich, MA, USA). *E. coli* BL21 (DE3) and Rosetta (DE3) pLysS were also purchased from Invitrogen Biotechnology Co. The genotypes of these strains are shown in Table 3.1.1.

Table 3.1 Genotypes of *E. coli* strains.

***E. coli* strain**	**Genotype**
Top10	*mcrA*, Δ(*mrr-hsd*RMS-*mcrBC*), Phi80*lacZ(del)M15*, Δ*lacX74*, *deoR*, *recA1*, *araD139*, Δ(*ara-leu*)7697, *galU*, *galK*, *rpsL(SmR)*, *endA1*, *nupG*
BL21(DE3)	*fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS* *λ DE3 = λ sBamHIo ΔEcoRI-B* *int: :(lacI::PlacUV5::T7 gene1) i21 Δnin5*
SHuffle	F′ *lac*, *pro*, *lacI*^{q} / *Δ(ara-leu)7697 araD139 fhuA2 lacZ:T7 gene1 Δ(phoA)PvuII phoR ahpC* galE (or U) galK λatt*::pNEB3-r1-*cDsbC* (Spec^{R}, *lacI*^{q}) *ΔtrxB rpsL150*(Str^{R}) *Δgor Δ(malF)3*
Rosetta 2 (DE3) pLysS	F^{-}*ompT hsdS*$_{B}$(r_{B}^{-} m_{B}^{-}) *gal dcm* (DE3) pLysSRARE2 (Cam^{R})

3.1.2 Plasmids

The pET28a vector with the ORF-ABC of the PUFA synthase from *Thraustochytrium sp.* ATCC 26185 was obtained from a previous study by Dr. Qiu's group (Figure 3.1). Expression vectors, such as pCDFduet, pETDuet and pCOLAduet were purchased from Promega Co. (CA, USA). pCDFDuet and pETDuet vectors were used as intermediate vectors for cloning individually open reading frame of the *Thraustochytrium* PUFA synthase.

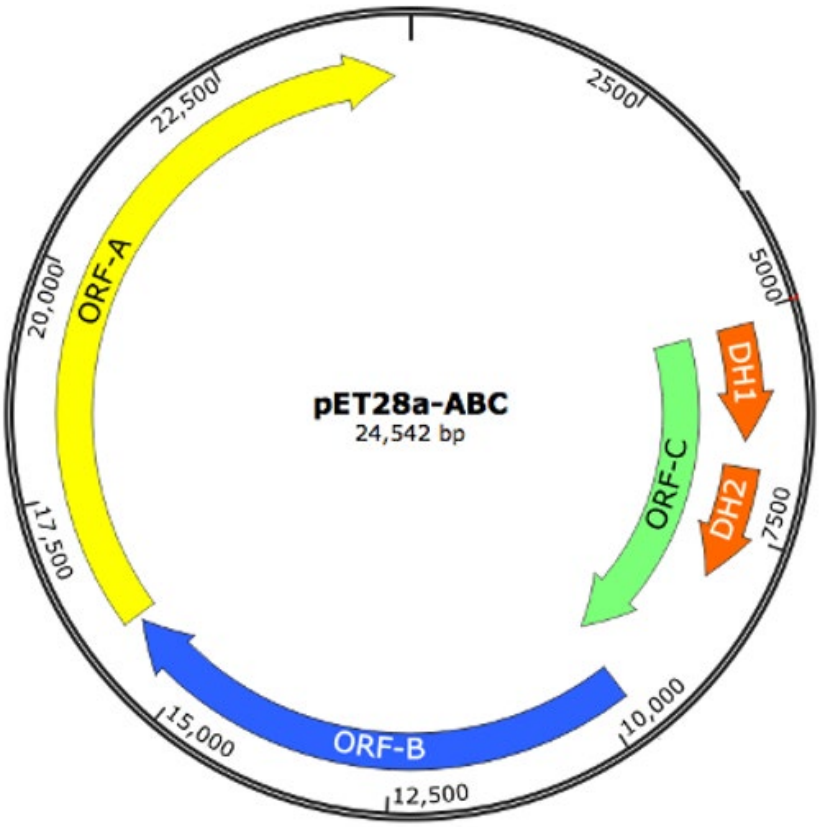


Figure 3.1 Physical map of pET28a expressing ORF-ABC of the PUFA synthase.

3.1.3 Luria Bertani (LB) medium and agar plates

LB medium was purchased from Thermo Fisher Scientific (Waltham, MA, USA) and prepared according the manufacturer instruction. For example, 25 g of LB medium powder were dissolved in 1 L of deionized water and then autoclaved at 120℃ for 20 min. For agar plates, 1 % of agar was added to the LB medium, autoclaved at the same condition and plated in 10 cm plastic plates. For antibiotic plates, final concentrations for kanamycin, ampicillin and spectinomycin were 50, 100 and 50 μg/mL, respectively.

3.1.4 Electro-competent cells

A single colony of each selected *E. coli* strain (Top10, BL21, Rosetta, and Shuffle) was inoculated into 5 mL of LB medium without antibiotics and incubated at 37℃ overnight. Three microliters of overnight culture were inoculated into 300 mL of LB medium and incubated at 37℃ until an OD_{600} 0.7-0.9 was reached. After that, the culture was cooled down on the ice and then centrifuged at 4℃ for 10 mins to pellet cells. The cell pellet was resuspended in 40 mL ice cold ddH_2O by gentle swirling and re-centrifuged at 4 ℃ for 15 mins. Another round of resuspended and centrifugation was performed with 20 mL ice-cold ddH_2O. After removing the supernatant, 4 mL of ice-cold 10% glycerol was used to re-suspend the cell pellet. The suspension was centrifuge again at 4℃ for 10 mins, and the cell pellet was resuspended in 600 μL ice cold 10% glycerol. Aliquots of 50 μL glycerol suspension were prepared and store at -80℃ for future transformation (Gonzales et al., 2013).

3.1.5 Enzymes and reagents for DNA amplification and purification

HP Taq DNA polymerase was purchased from Bio Basic Inc. Q5 polymerase and restriction enzymes, and dNTP were purchased from New England Biolabs (Ipswich, MA, USA). Primers were synthesized through Sigma-Aldrich (St. Louis, MO, USA). T4 ligase was acquired from Thermo Fisher Scientific. DNA purification kit and PCR fragment cleanup kit were purchased from Bio Basic Inc. (York, ON, Canada).

3.2 Sequence analysis of the PUFA synthase in *Thraustochytrium*

The sequence of the polyunsaturated fatty acid synthase from *Thraustochytrium* sp. ATCC 26185 (GenBank accession no. AOG21005.1) was analyzed by bioinformatics tools, which included BLASTp (NCBI), MegAlign (DNASTAR), and SWISS-MODEL. The multisequence alignment of homologous sequences from *Schizochytrium*, *Moritella*, *Shewanella* and *E. coli* by DNASTAR was used to identify actives sites and putative important residues. The homology model of DH1 And DH2 was built by on-line homology modeling tools provided by the SWISS-MODEL using FabA from *E. coli* as a template.

3.3 Homology model of DH1 domain from subunit-C of PUFA synthase

The putative 3D structure of DH1 and DH2 domains in ORF-C was built using FabA as a template. FabA possessed a "hot dog" structure fold with a core α-helix surrounded by six antiparallel strands of β-sheets. In *E. coli* FabA, catalytic residues are located at 70 (histidine) and 84 (aspartic acid). Two phenylalanine residues at 21 and 92 located at the end of the substrate binding pocket were shown to play important role in determining the substrate specificity (Finzel et al., 2015). Phenylalanine at 260 and methionine at 337 was identified as the corresponding residues in DH1 domain of the PUFA synthase.

3.4 Site-directed mutagenesis of residues in DH domains

Overlapping PCR was employed for site-directed mutagenesis of key residues for catalysis in DH domains. Two sequential PCR reactions using two pairs of primers were used to introduce the mutation of interest in DH domains (Table 3.4). A DNA fragment of ORF-C was released from pET28a-ABC using the restriction enzymes *Eco*RV and *Nde*I, and sub-cloned into pETDuet using the following ligation condition: 2 μL of 5×ligation buffer, 1 μL pETDuet backbone, 6.5 μL ORF-C fragment and 0.5 μL Invitrogen T4 DNA ligase at 16ºC overnight. The ligation product was transformed into *E. coli* by electroporation. The recombinant plasmid carrying ORF-C was isolated from the transformants verified by colony PCR and digested with BamHI to release a fragment encoding DH1 and DH2 domains. The fragment was sub-cloned into pCDFDuet digested by *Bam*HI for site-directed mutagenesis. The recombinant plasmid pCDFDuet-DH1-DH2 carrying wild-type DH1 and DH2 domain fragment was then used as a template for two sequential PCR for site-directed mutagenesis. Two overlapping sequences with mutagenized sites were amplified separately by the first PCR using one internal primer and one flanking primer. The second PCR was undertaken using mixed two DNA fragments as a template and two flanking primers. The resulting mutagenized fragment was digested with an appropriate restriction enzyme and sub-cloned into pETDuet or pET28a. The recombinant plasmids were confirmed by restriction digestion and DNA sequencing, and confirmed plasmids were transformed into *E. coli* strains.

Table 3.4 Primers used for site-directed mutagenesis. Mutation site is underlined.

Name	Sequence (5'→ 3')
DH1-H315A F	CTTTCCCTGCGCCTTCGTCAAGGACGAGGTCA
DH1-H315A R	CTTGACGAAGGCGCAGGGAAAGTACCAGTGGTC
DH2-H288A F	CTTCGCGTGCGCCTTCTGGTTCGACTCGGTCA
DH2-H288A R	GAACCAGAAGGCGCACGCGAAGAACCAGTCGT
DH1-F260A F	CCTCGGTCGCCGGCTCCAA
DH1-F260A R	TTGGAGCCGGCGACCGAGG
DH1-M337A F	ATGCTCAAGGCGTACATGATCTGGCT
DH1-M337A R	AGCCAGATCATGTACGCCTTGAGCAT

3.5 Reconstruction of a PUFA synthase containing mutated DH domains in ORF-C

To reconstruct a PUFA synthase containing an ORF-C with mutated DH domains, the original DH domains in ORF-C in pETDuet were replaced by mutated DH domains using BamHI restriction digestion and ligation. Restriction digestion was performed in a 50μL reaction containing up to 1 μg DNA, 5 μL 10×reaction buffer, 10 units of *Bam*HI and nuclease-free water. Ligation was performed in a 10 μL reaction containing insert and vector DNA fragments with a 3:1 ratio, 2 μL 5×reaction buffer, 0.5 μL T4 ligase and nuclease-free water. The reconstituted ORF-C released from the plasmid by *Eco*RV and *Nde*I digestion was then used to replace the original ORF-C in pET28a-ABC.

3.6 Expression of mutated DH domains of PUFA synthase in *E. coli*

To reconstruct a PUFA synthase with mutated DH domains, the ORF-C with mutated DH domains were individually placed back into pET28a-ABC. The confirmed plasmids pET28a-DH1-H315A-DH2, pET28a-DH1-DH2-H288A, pET28a-DH1-H315A-DH2-H288A, pET28a-DH1-F260A-DH2, pET28a-DH1-M337A-DH2 were transformed into *E. coli* BL21 (DE3) competent cells. A single colony of the transformant was inoculated to 5 mL of LB medium with kanamycin and incubated overnight at 37ºC. An aliquot of 200 μL of the overnight culture was then inoculated into 20 ml LB liquid medium and incubated at 37ºC until an OD_{600} of 0.6 reached. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the final concentration of 0.5 mM for inducing the expression at 16℃ for six days. After that, the biomass was collected for fatty acid analysis.

3.7 Construction of a PUFA synthase containing ORF-C with a deleted DH domain

3.7.1 Construct a PUFA synthase containing ORF-C with deleted DH1 domain

To construct a PUFA synthase containing ORF-C with a deleted DH1 domain, the following procedures were followed. First, pCDFDuet plasmid was digested with *BgL*II and treated with a large Klenow fragment of DNA polymerase I, which filled in the 5' overhangs for blunt end ligation. On the other hand, a fragment coding for two DH domains (DH1 and DH2) in ORF-C was released from pETDuet-C by *Bam*HI digestion and cloned into pCDFDuet, giving pCDFDuet-DH1-DH2. Inverse PCR was then conducted using the pCDFDuet-DH1-DH2 plasmid as a template and two outward specific primers (Table 3.5) with StuI restriction sites. The PCR product was self-ligated according to the standard procedure and transformed into competent *E. coli* Top10 cells by electroporation. The resultant plasmid pCDFDuet-DH1$^{\Delta}$-DH2 was then digested with restriction enzyme *Bam*HI. The released fragment was inserted into the same restriction site of the original pETDuet-C, giving pETDuet-C*(DH2). The ORF-C* with deleted DH1 domain was released by digestion with *Eco*RV and *Nde*I and ligated into pET28a-ABC backbone with the same restriction digestion. The ligates, C*-DH2/pET28a-AB, were transformed into electro-competent *E. coli* Top 10 cells by electroporation. Transformants were selected on the selection plates with incubation at 37ºC overnight. Positive transformants were selected, sub-cultured, and verified using PCR screening using a universal primer and a gene-

specific primer. The plasmid pET28a-ABC*(DH2) of the positive transformant was extracted using BioBasic's miniprep kit and confirmed by sequencing.

3.7.2 Construct a PUFA synthase containing ORF-C with double DH2 domain

To construct a PUFA synthase with double DH domains where DH1 was replaced with DH2, the DH2 fragment was amplified using pETDuet-C as a template and two primers (BglII-DH2-F/ BgLII-DH2-R). Amplified DH2 fragment was digested with *BgL*II and inserted into the BglII site of pCDFDuet-DH1$^{\Delta}$-DH2, giving pCDFDuet-DH2-DH2. The double DH2 fragment was then released from the plasmid by *Bam*HI digestion and inserted into the same site of pETDuet-C, giving pETDuet-C*(DH2-DH2). Next, pETDuet-C*(DH2-DH2) was digested with *Eco*RV and *Nde*I; the released fragment was inserted into pET28a-ABC digested with the same restriction enzymes, giving pET28a-ABC*(DH2-DH2).

3.7.3 Construct a PUFA synthase containing ORF-C with deleted DH2 domain

To delete the DH2 domain of ORF-C in PUFA synthase, a similar strategy, as described above, was used. First, pCDFDuet plasmid was digested with *Nco*I, and the cohesive end was filled using the Klenow fragment of DNA polymerase I. On the other hand, a fragment coding for two DH domains (DH1 and DH2) of ORF-C in pETDuet-C was released by *Bam*HI and cloned into the same site of pCDFDuet, giving pCDFDuet-DH1-DH2. Inverse PCR was conducted to amplify the plasmid pCDFDuet-DH1-DH2$^{\Delta}$. Then, the fragment for the DH1 domain was placed back into pETDuet-C at the *Bam*HI site. The ORF-C* containing deleted DH2 domain was released by digestion with *Eco*RV and *Nde*I and inserted into pET28a-ABC digested with the same enzymes, giving pET28a-ABC*(DH1).

Table 3.7 Primers used in construction of single DH1, single DH2 and double DH2.

Name	Sequence (5' ⟶ 3')	Annotation
Add-StuI F	CCTATGACCTGGCACCCCAT	Half StuI site (underline)
Add-StuI R	CCTGCGCGAATTCGGATCCTGG	Half StuI site (underline)
NcoI-DH1 F	CATCCATGGCGATGGCGCTCCG	NcoI restriction site (underline)
NcoI-DH1 R	TTGGCGAAGAGCGAGGTGC	
BgLII-DH2 F	GAAGATCTGCATGTCCCGCCTCGGCGA	BgLII restriction site (underline)
BgLII-DH2 R	GGTGCCGGTGACGATGAAG	

3.8 Expression of a PUFA synthase with deleted DH domains in *E. coli*

To reconstruct a PUFA synthase with mutated DH domains, the ORF-C with modified DH domains were placed back into pET28a-ABC, giving plasmids with reconstituted PUFA synthases. The confirmed plasmids pET28a-ABC*(DH2), pET28a-ABC*(DH2-DH2), and pET28a-ABC*(DH1) were transformed into *E. coli* BL21 (DE3) competent cells. A single colony of transformants was inoculated to 5 mL of LB medium with kanamycin and incubated at 37℃ for overnight. An aliquot of 200 μL of the overnight culture was then inoculated into 20 mL LB liquid medium and incubated at 37℃ until an OD_{600} of 0.6 reached. IPTG was added to the final concentration of 0.5 mM for inducing the expression at 16℃ for six days. After that, the cells were harvested for fatty acid analysis.

3.9 Fatty acid analysis

Fatty acid profiles of the cell cultures were analyzed by gas chromatography (GC) analysis. Total fatty acids in *E. coli* were converted to fatty acid methyl esters (FAMEs) by the reaction with 2 mL of 1% sulfuric methanol at 80℃ for 1 hour. After that, the samples were cooled down on the ice and added with 1 mL 0.9% NaCl and 2 mL hexane. The mixture was centrifuged at 2,200 rpm for 5 mins for phase separation. The hexane phase containing FAMEs was transferred to a new tube and dried under N_2 gas. After drying, the sample was resuspended in an appropriate amount of hexane and used for GC analysis by an Agilent 7890A system with

a DB-23 column installed. Chromatographic peaks of FAME components were identified and quantified in reference to Supelco 37 Component FAME Mix (Sigma, USA) and an internal standard (C17:0).

3.10 PUFA synthase protein purification

3.10.1 Expression of the wild type PUFA synthase in *E. coli*

Three ORFs of the wild type PUFA synthase originally cloned into pET28a vector under a strong T7 promoter (Figure 3.1) that can be induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) was made available to this research. The plasmid was transformed into *E. coli* BL21 strain by electroporation as follows. One μL of the plasmid solution was mixed with the competent cells, which was immediately transferred into the ice-cold cuvette. The cuvette was placed on ice for about 2 minutes, and the moisture outside the cuvette was wiped out gently with Kim-wipe before it was put into the electroporator (Fiedler & Wirth, 1988). Electroporation was conducted under the condition of 1800 V with two pulses. After electroporation, 1 mL LB medium was added into the cuvette, and the mixture was transferred to a new tube. After incubation at 37℃ for one hour, the mixture was then spread on LB agar plate with 50 μg/μL kanamycin antibiotic and incubated at 37℃ for overnight. Transformants grown on the selection plate were verified by colony PCR. The positive colony was selected and stored in -80 ℃ freezer with 25% glycerol as final concentration.

3.10.2 Optimization of the PUFA synthase expression

To optimize the PUFA synthase expression, a variety of independent parameters such as different *E. coli* hosts, types of culture media, inducer concentration, induction temperature and post-induction incubation time were tested.

3.10.2.1 Expression of the PUFA synthase in different *E. coli* hosts

The Expression of the PUFA synthase in three different *E. coli* strains was initially investigated. For this purpose, two plasmids pET28a-ORF-ABC and pCDFDuet-PPTase were co-transformed into BL21(DE3), Rosetta 2 (DE3) pLysS and SHuffle hosts. The transformed cells were plated in LB agar containing 50 μg/μL of kanamycin and 50 μg/μL of spectinomycin. After

overnight incubation, a single colony of transformants was grown overnight in 3 mL of LB medium containing the mentioned antibiotics on a rotary shaker (200 rpm) at 37ºC. On the next day, 10 mL of LB-antibiotics medium was inoculated by 100 μL of the pre-culture and incubated under the same conditions. Once the culture reached the mid-exponential phase (OD_{600} at ~0.6), induction of protein expression was performed with 0.5 mM IPTG at 16ºC for 36 hours. After that, the cells were harvested by centrifugation at 6000 rpm at 4ºC for 10 mins and resuspended in 0.5 mL of 1×phosphate-buffered saline (PBS) buffer at PH 7.4 with 5 mM phenylmethylsulfonyl fluoride (PMSF). Bead beater was used to disrupt the suspended cells under the following condition: for each 5 mL aliquot of suspension, beating for 30 sec, and then on ice for 1 min, repeating three times. The lysate was centrifugated at 4ºC for 15 mins at 12,000 rpm. After centrifugation, the supernatant of each sample was collected for examining the expression level of the PUFA synthase by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

3.10.2.2 Expressing of the PUFA synthase in different culture media

To determine the effect of different culture media on the yield of the recombinant proteins, three types of media (LB, LB with 10% glycerol and 2×YT with 10% glycerol) were used to grow BL21 (DE3) transformants harboring pET28a-ORF-ABC and pCDFDuet-PPTase in 3 mL initially. Then, 20 mL of each medium was inoculated with the pre-culture with a ratio of 1:100. When OD_{600} reached 0.6, the cultures were induced with 0.5 mM of IPTG at 16ºC for 36 hours. After that, the induced cells were harvested for examining the expression level of the PUFA synthase proteins, as described above.

3.10.2.3 Expressing of the PUFA synthase in different inducer concentration

BL21 (DE3) cells containing the two plasmids were grown at 37ºC overnight in LB medium with antibiotics. A 200 μL of the pre-culture was then inoculated into 20 mL of 2×YT/10% glycerol medium at the ratio of 1:100 and incubated at 37ºC. When the OD_{600} of cultures reached 0.6, induction was performed by adding IPTG at the concentrations of either 0.5 or 1 mM. After 36 h of incubation at 16ºC, the cells were harvested for examining the expression level by SDS-PAGE.

3.10.2.4 Expressing of the PUFA synthase in different post-induction incubation time under different temperature

The effect of four different post-induction incubation times (12, 24, 36 and 48 hours) under three different incubation temperatures (22ºC, 30ºC and 37ºC) was also evaluated for the expression of the PUFA synthase. Similarly, a small amount of BL21 (DE3) pre-culture was inoculated into 30 mL of 2×YT/10% glycerol medium with antibiotics. When the culture reached OD_{600} at 0.6, the induction was started by adding IPTG at a final concentration of 1 mM. After that, the cultures were incubated at three different temperatures, 22°C, 30°C, and 37°C. At four different time points, 12, 24, 36 and 48 hours, 5 mL of each culture was withdrawn for the analysis of the expression.

3.10.3 Purification of the PUFA synthase proteins

Each of the three subunits of the PUFA synthase has an N-terminal His-Tag. Therefore, the Nickel-NTA resin could be used for purifying all the PUFA synthase subunits. For protein purification, 300 mL of BL21 (DE3)-pET28a-ABC culture was grown at 1 mM IPTG and 16°C for 12 h. The cells were pelleted by centrifugation, disrupted by bead-beating and resuspended in a 3 mL His-binding buffer with 1mM PMSF and resins (Table 3.10.3). After centrifugation at 12,000 rpm, 4°C for 15 mins, supernatants were transferred into the washed resin tubes and incubated on the Rocker-shaker at room temperature for 30 mins. After incubation, the pellets were collected by centrifugation, and washed three times by the His-washing buffer. After that, 50 μL of a His-elution buffer with PMSF was added to pellets to elute the proteins by incubation at room temperature on the rocker for 30 mins (Bornhorst & Falke, 2000). After centrifugation, the supernatant with partially purified proteins was filtered through a 0.45μm filter. The collected solution was used for protein analysis. The concentration of proteins was measured by Bradford assay (Kruger, 1994).

Table 3.10 Buffers used for His-Tag protein purification.

	Tris-HCl (PH 8.0)	NaCl	EDTA	Imidazole
His-binding buffer	50 mM	100 mM	0.1 mM	5 mM
His-washing buffer	50 mM	300 mM	0.1 mM	10 mM
His-elution buffer	50 mM	50 mM	0.1 mM	300 mM

3.10.4 Western blot analysis

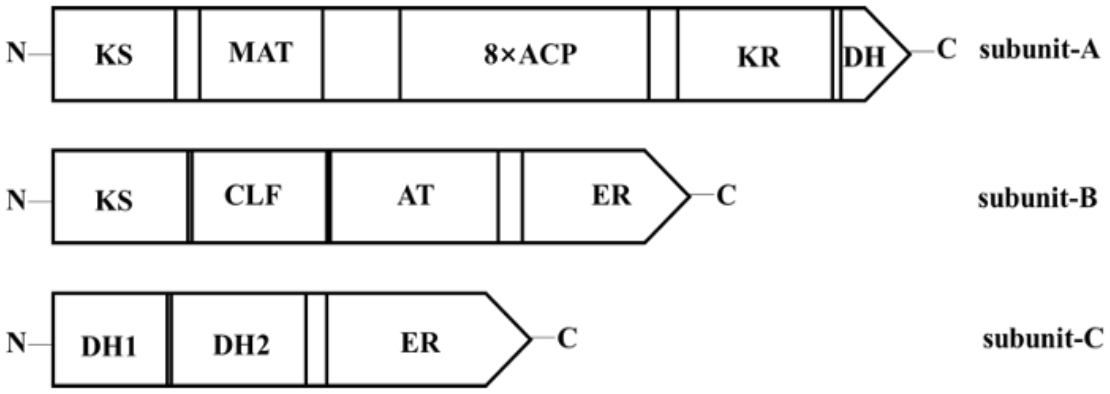
For Western blot analysis, protein samples were adjusted to the final concentration of 1.0 or 1.5 μg/μL. Fifteen μL of protein samples were added with 5 μL 4×sample buffer with 2-mercaptoethanol, which was then denatured by heating at 95°C for 5 min. After cooling down on the ice, samples were centrifuged at 15,000 rpm for 10 min. The supernatants were loaded on a polyacrylamide gel with a 6% polyacrylamide stacking gel and a resolving gel. The electrophoresis was conducted in 1×running buffer (25mM Tris base, 192mM glycine and 0.1% SDS) at 100 to 150V. After electrophoresis, fractionated proteins in the gel were transferred to a nitrocellulose membrane in 1×transfer buffer (25 mM Tris base, 192 mM glycine and 20% methanol) using Trans-Blot Turbo Transfer system at 25V for 7 mins. The membrane was blocked in 3% skim milk powder in phosphate-buffered saline with 0.05% Tween® 20 (PBST) at 37°C for 2 h, followed by washing three times in 3% skim milk/PBST buffer. After washing, the membrane was incubated with an anti-His Tag mouse monoclonal antibody solution (1:1000 dilution) at 37°C for 2 hours (Mahmood & Yang, 2012). After that, the membrane was washed two times with PBST and incubated with a DAB (3,3'-diaminodbenzidine) substrate working solution at 37°C for 10 min to visualize the proteins.

4. RESULTS

4.1 Sequence analysis of DH domains in the PUFA synthase

The PUFA synthase from *Thraustochytrium* sp. ATCC 26185 for the biosynthesis of VLCPUFAs comprises three subunits encoded by three large open reading frames, each with multiple catalytic domains. These catalytic domains were identified in the presence of typical catalytic motifs (Meesapyodsuk & Qiu, 2016). Two FabA-like DH domains were located in the N-terminal region of subunit-C (DH1 and DH2) of the PUFA synthase and shared 32% and 27% of amino acid identity to *E. coli* dehydratase FabA, respectively. However, the two domains shared only about 15% of amino acid identity with each other (Fig. 4.1A). Two predicted catalytic residues, histidine and aspartic acid/glutamic acid were found in the middle of each domain. Two other corresponding residues, such as F260 and M337 previously shown to have important roles in interacting with substrates of FabA, were also identified in the DH1 domain (Figure 4.1B). Homology modelling of the two DH domains based on the FabA structure revealed that although the two DH domains had a low sequence similarity, both possessed a similar double "hot dog" structure; each hotdog was comprised of a core helix surrounded by two β-sheets. The conserved catalytic histidine residue was located in the loop region prior to the start of the central "hot dog" helix, while the conserved aspartic acid/glutamic acid residue resided on the hot dog helix (Fig 4.1C). Phylogenetic analysis indicated that DH1 domains from both prokaryotic and eukaryotic PUFA synthases could be clearly clustered into one group, which was closer to *E. coli* FabA. (Fig. 4.1D).

(A)


N
KS
MAT
8×ACP
KR
DH
C
subunit-A
N
KS
CLF
AT
ER
C
subunit-B
N
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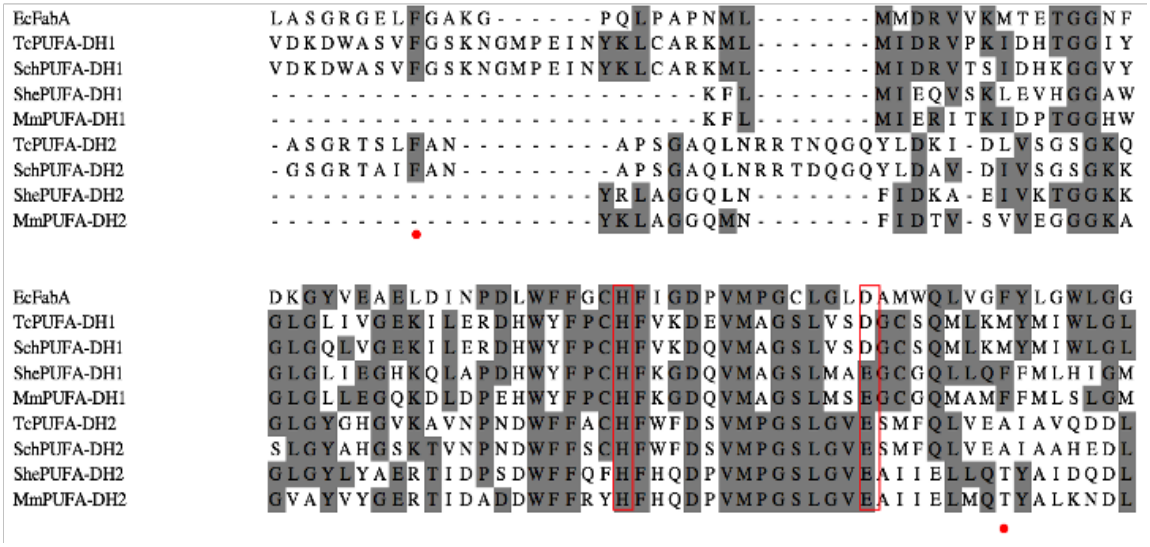
ER
C
subunit-C

(B)


EcFabA
TcPUFA-DH1
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MmPUFA-DH1
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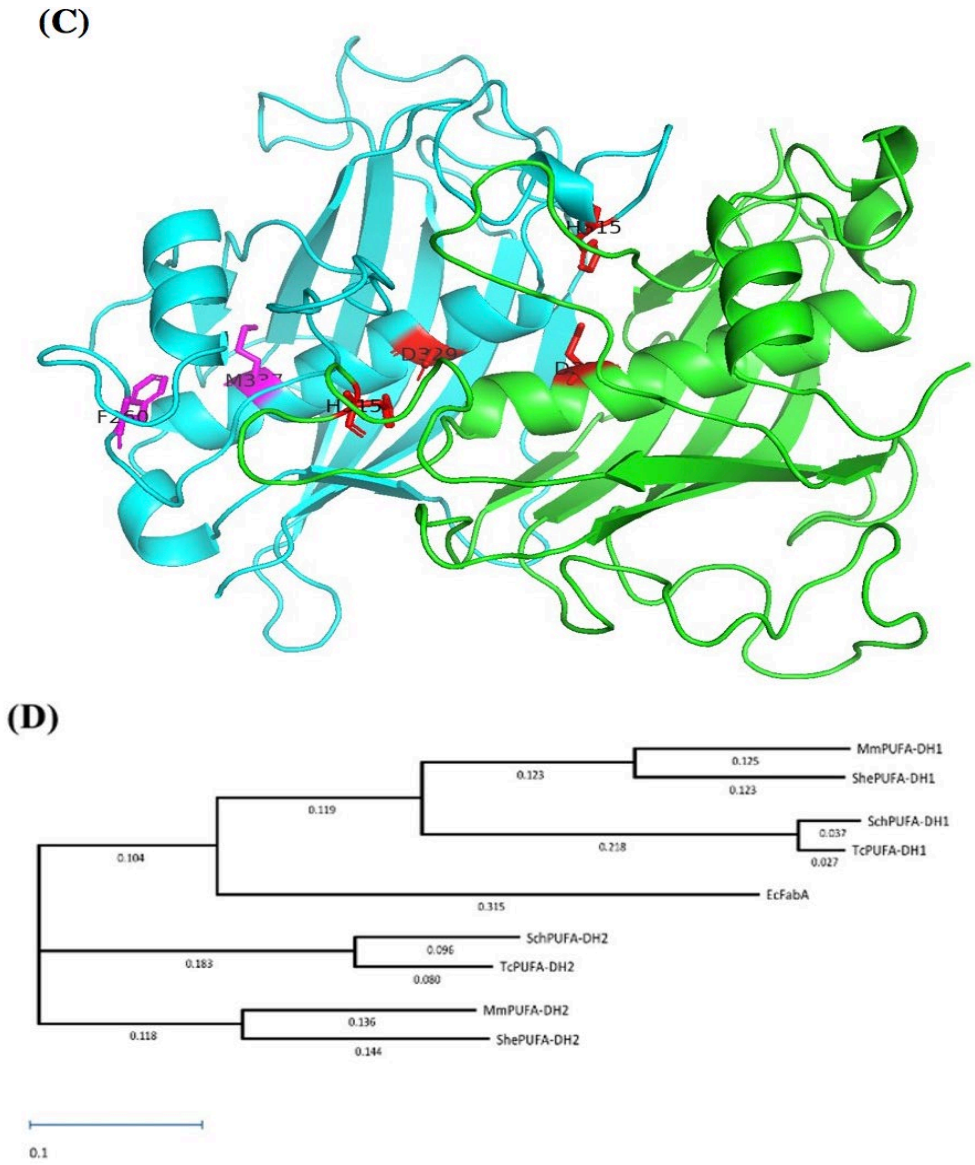

Figure 4.1 Domain organization and sequence analysis of two tandem FabA-like DH domains of the PUFA synthase from *Thraustochytrium*. (A) Domain organization of the PUFA synthase from *Thraustochytrium* sp. ATCC 26185. (B) Multiple sequence alignment of DH domains from PUFA synthases and FabA from *E. coli*. (C) Homology model of DH1 domain in subunit-C with labelled residues (D) Phylogenetic analysis of the resulting alignment. ACP, acyl carrier protein; KS, 3-ketoacyl-ACP synthase; KR, 3-ketoacyl-ACP reductase; DH, 3-hydroxyacyl-ACP dehydratase; ER, enoyl-ACP reductase. EcFabA, FabA from *E. coli* (Heath and Rock, 1996); TcPUFA-DH1, DH1 domain from *Thraustochytrium* PUFA synthase; TcPUFA-DH2, DH2 domain from *Thraustochytrium* PUFA synthase (Meesapyodsuk and Qiu, 2016); SchPUFA-DH1, DH1 domain from *Schizochytrium* PUFA synthase; SchPUFA-DH2, DH2 domain from *Schizochytrium* PUFA

synthase (Ye et al., 2015); MmPUFA-DH1, DH1 domain from *Moritella* PUFA synthase; MmPUFA-DH2, DH2 domain from *Moritella* PUFA synthase (Kautharapu & Jarboe, 2012); ShePUFA-DH1, DH1 domain from *Shewanella* PUFA synthase ShePUFA-DH2, DH2 domain from *Shewanella* PUFA synthase (Okuyama et al., 2007).

4.2 Functional analysis of mutagenized DH domains of PUFA synthase in *E. coli*

To investigate the function of two DH domains in ORF-C of the PUFA synthase, a DNA fragment encoding the two DH domains was first analyzed by site-directed mutagenesis. For doing this, the whole ORF-C was released from pET28a-ABC (Meesapyodsuk & Qiu, 2016) by a double digestion of *Eco*RV and *Nde*I, and sub-cloned into pETDuet where a single *Bam*HI site was eliminated by cutting, filling, and blunt-end ligation, giving pETDuet-C. The fragment coding for two adjacent DH domains (DH1 and DH2) in the N-terminus of ORF-C was then released from pETDuet-C by *Bam*HI digestion and cloned into pCDFDuet, giving pCDFDuet-DH1-DH2. Subsequent site-directed mutagenesis on individual DH domains was carried out using the plasmid as templates. The histidine residue at the active site of each DH domain were substituted by an alanine using overlapping PCR. After mutation, individual DH domains were placed back into BamHI site of pETDuet-C. To reconstitute a PUFA synthase comprising three ORFs with site-mutagenized DH domains in one operon, the ORF-C* containing DH site mutations was released from pETDuet-C* by *Eco*RV and *Nde*I digestion and inserted into pET28a-ABC digested with the same two enzymes, giving pET28a-ABC* (Figure 4.2.1).

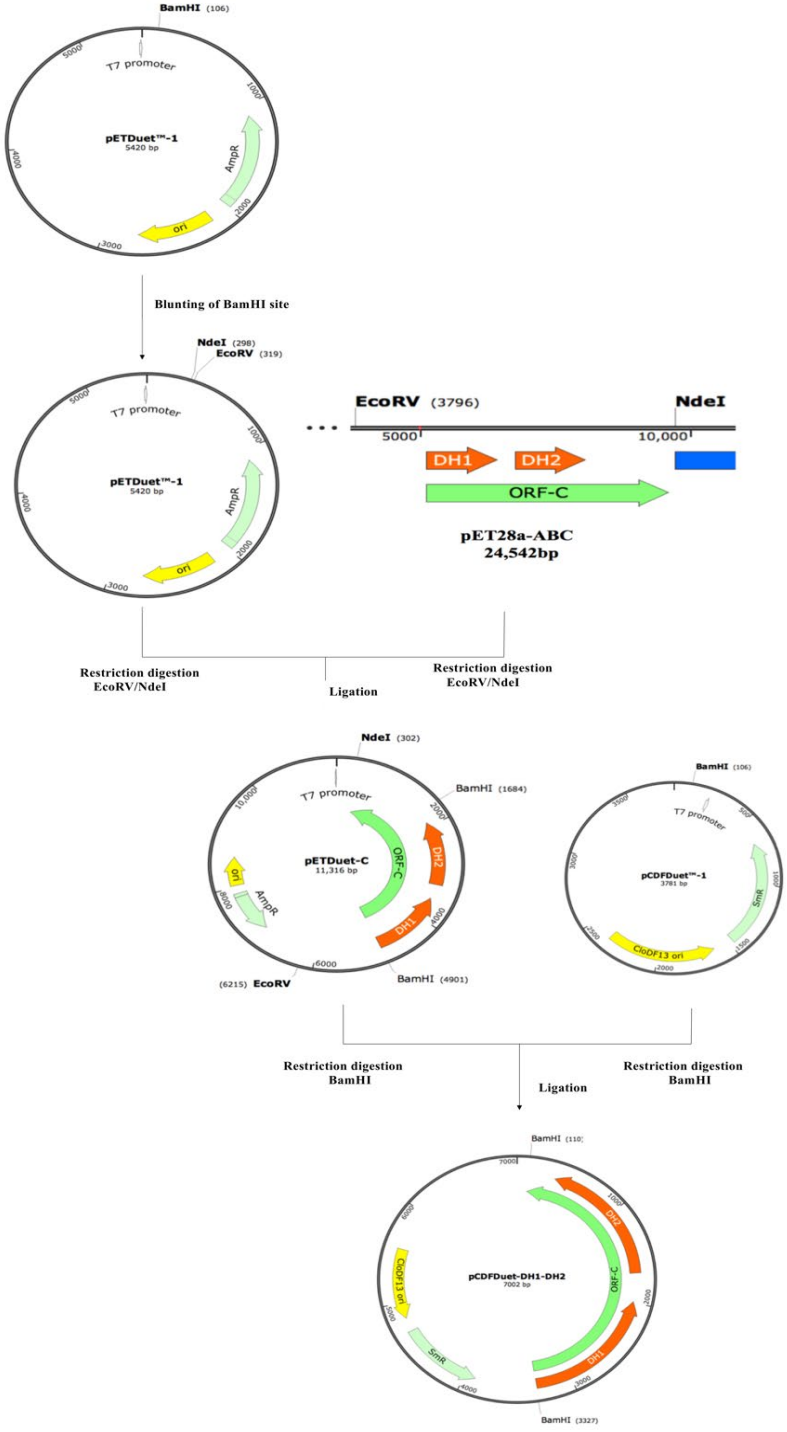

Figure 4.2.1 Construction of the recombinant plasmid for site-directed mutagenesis.

To functionally analyze the effect of site-mutagenized DH domains, pET28a-ABC* was expressed along with a plasmid carrying a gene encoding phosphopantetheinyl transferase from Nostoc PCC7120 (HetI) in *E. coli* BL21. The transformants were grown at 16℃ with 1 mM IPTG to induce the expression of the mutated PUFA synthases. As shown in Figure 4.2.2, three bands with the size of 295 kDa, 223 kDa and 165 kDa on a SDS-PAGE gel corresponding to three subunits of the mutated PUFA synthases were clearly observed after 12 hours of induction. It appeared that the expression level of subunit-C was the highest, followed by those of subunit-B and subunit-C in all transformants expressing mutated PUFA synthases. This western blot result confirms all reconstituted PUFA synthase with mutated DH domains were successfully expressed.

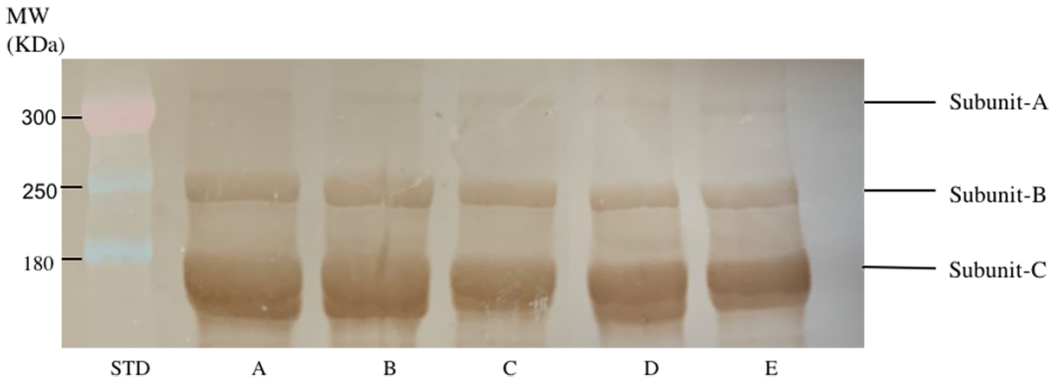

Figure 4.2.2 Western blot analysis of expressions of the PUFA synthase with mutated DH domains in *E. coli*. All constructs were expressed in *E. coli* BL21. Lane A: pET28a-ORF-ABC-DH1-F260A; Lane B: pET28a-ORF-ABC-DH1-M337A; Lane C: pET28a-ORF-ABC (positive); Lane D: pET28a-ORF-ABC-DH1-H315A; Lane E: pET28a-ORF-ABC-DH2-H288A; 10μg of purified protein was loaded in each lane.

Fatty acid analysis of transformants showed that substitution of the histidine residue with alanine in DH1 (TcPUFAs-DH1-H315A-DH2) gave the same fatty acid profile as the empty vector control in *E. coli*, without any VLCPUFAs produced. Substitution of the histidine residue with alanine in DH2 (TcPUFAs-DH1-DH2-H288A) gave a fatty acid profile with a small amount of DPA, but not DHA, which was different from wild type PUFA synthase control where both DPA

and DHA were produced. A double substitution of the histidine residues in both DH1 and DH2 (TcPUFAs-DH1-H315A-DH2-H288A) gave the same fatty acid profile as the vector control in *E. coli*, with no VLCPUFAs produced (Figure 4.2.3). This result indicates that DH1 domain, but not DH2 domain of the PUFA synthase, is essential for the biosynthesis of VLCPUFAs, and DH2 domain is required for the synthesis of DHA. In addition, substitutions of the phenylalanine and methionine residues in DH1 domain presumably at the bottom of the substrate tunnel with alanine (F260A and M337A) did not result in observable changes in fatty acid profiles (data not shown), implying that these two amino acids may not be critical for catalytic activity and substrate interaction.

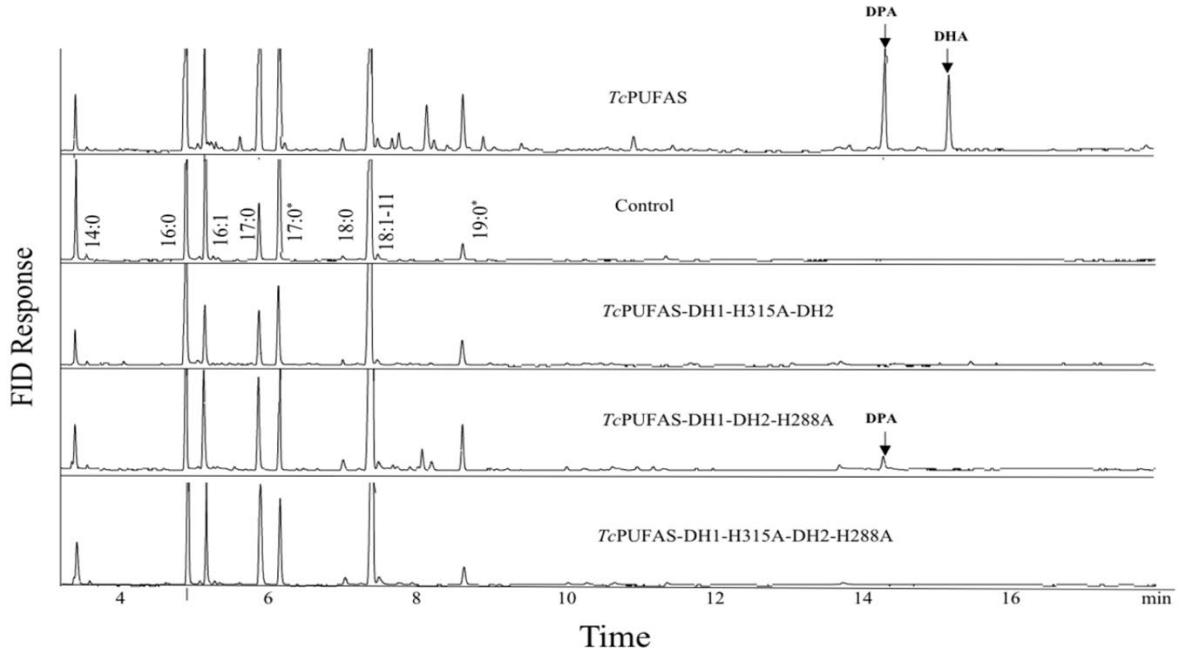

Figure 4.2.3 GC analysis of *E. coli* transformants expressing PUFA synthases with site-mutagenized DH domains. TcPUFAS: *E.coli* expressing a wild type PUFA synthase; Control, *E.coli* expressing a empty vector; TcPUFAs-DH1-H315A-DH2, *E.coli* expressing a PUFA synthase with the mutated DH1 domain; TcPUFAs-DH1-DH2-H288A, *E.coli* expressing a PUFA synthase with the mutated DH2 domain; TcPUFAs-DH1-H315A-DH2-H288A, *E. coli* expressing a PUFA synthase with mutated DH1 and DH2 domain.

4.3 Functional analysis of deleted DH domains of PUFA synthase in *E. coli*

The above site-directed mutagenesis analysis of DH domains revealed the distinct functions of two FabA-like DH domains of the PUFA synthase in the biosynthesis of VLCPUFAs. To confirm the result, we reconstructed the PUFA synthase with deleted individual DH domains for functional analysis. DH domain deletions were carried out on the plasmid using inverse PCR. For deleting DH1 domain, two inverse PCR primers flanking the DH1 domain was used for PCR, and amplified products were self-ligated, giving pCDFDuet-DH1$^{\Delta}$-DH2 (Figure 4.3.1). For deleting DH2 domain, two inverse PCR primers flanking the DH2 domain was used for PCR, and amplified products were self-ligated, giving pCDFDuet-DH1-DH2$^{\Delta}$. Considering the possibility that two adjacent DH domains might possess structure-supporting roles of each other, a double DH domain construct where DH1 was replaced by DH2 was also made by amplification and insertion of a second copy of DH2 into BglII of pCDFDuet-DH1$^{\Delta}$-DH2, giving pCDFDuet-DH2-DH2. Afterwards, these DH domain deletion and swapping were released by BamHI digestion and placed back into pETDuet-C at the same site. The ORF-C* containing deleted and swapped DH domains was released from pETDuet-C* by *Eco*RV and *Nde*I digestion and inserted into pET28a-ABC* where three ORFs of a PUFA synthase with mutated DH domains were assembled into one operon for functional analysis in *E. coli* (Figure 4.3.2).

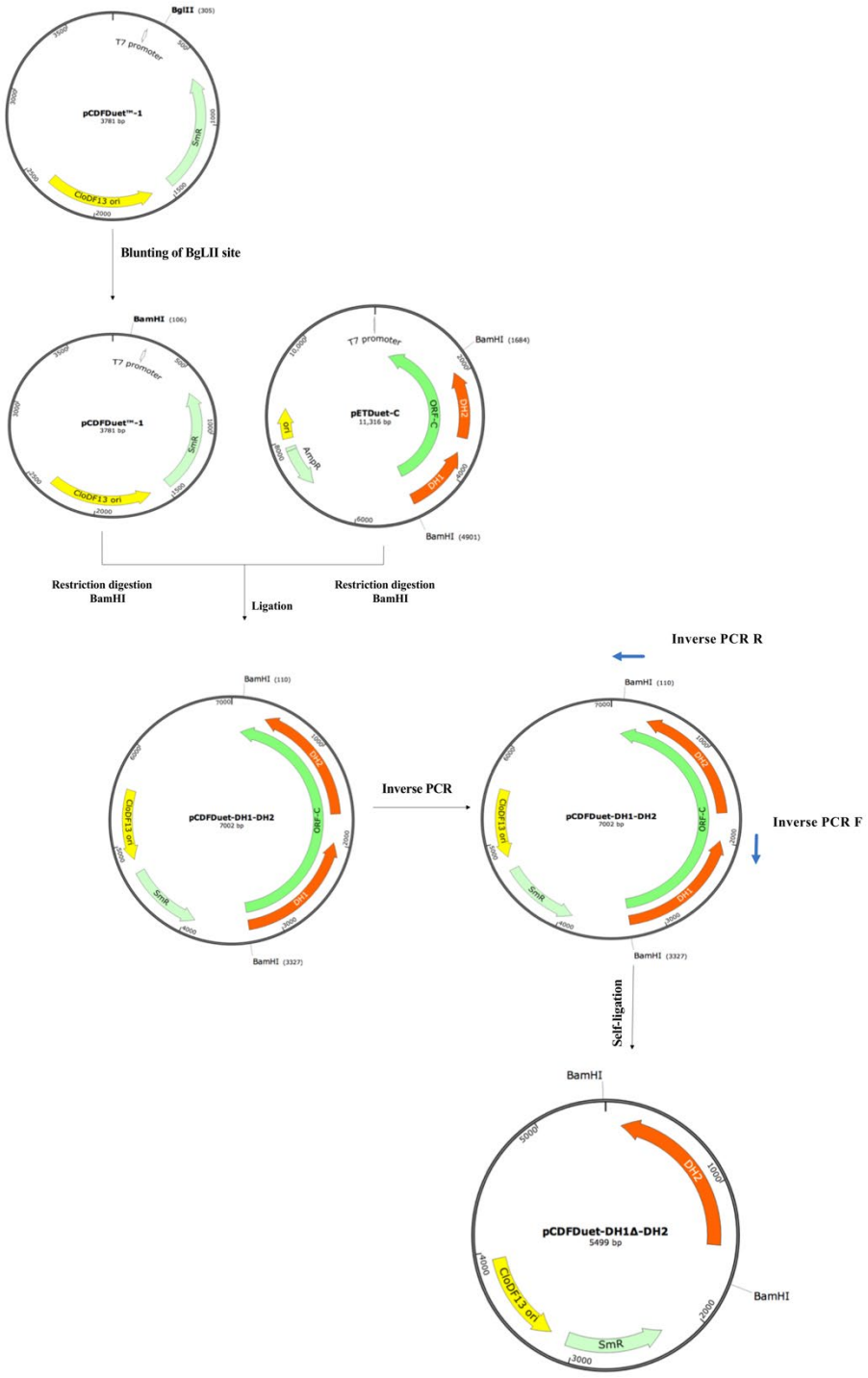

Figure 4.3.1 Construction of the plasmid with deleted individual DH domain.

Figure 4.3.2 Construction of the plasmid expressing an operon of the PUFA synthase with double DH2 domains.

As shown in Figure 4.3.3, deleting the DH1 domain from the PUFA synthase (TcPUFAs-DH2) produced the same fatty acid profile as the vector control, without any VLCPUFAs produced. Deleting the DH2 domain from the PUFA synthase (TcPUFAs-DH1) produced a fatty acid profile with a small amount of DPA, but not DHA. This result completely mirrored site-directed mutagenesis analysis of individual DH domains described above. In addition, replacement of DH1 with DH2 domain (TcPUFAs-DH2-DH2) gave a fatty acid profile, similar to a single DH2 domain, with no VLCPUFAs produced. These results further confirm that DH1 domain, but not DH2 domain, is essential for the biosynthesis of VLCPUFAs.

As a single or double DH2 domain of the PUFA synthase was unable to synthesize any VLCPUFAs while a single DH1 domain retained some activity to synthesize DPA, we then compared fatty acid productions of *E. coli* transformants expressing PUFA synthases with site-mutagenized DH2 domain and deleted DH2 domain. As shown in Table 4.3, site-mutagenized DH2 domain resulted in the drastic reduction of DPA production by about 95%, while deletion of DH2 domain resulted in even more reduction of DPA production by about 97%, as compared to the wild type. This result indicates that deletion of the DH2 domain might have the disrupting effect on the structure of the PUFA synthase, thereby resulting in even lower activity in the biosynthesis of DPA.

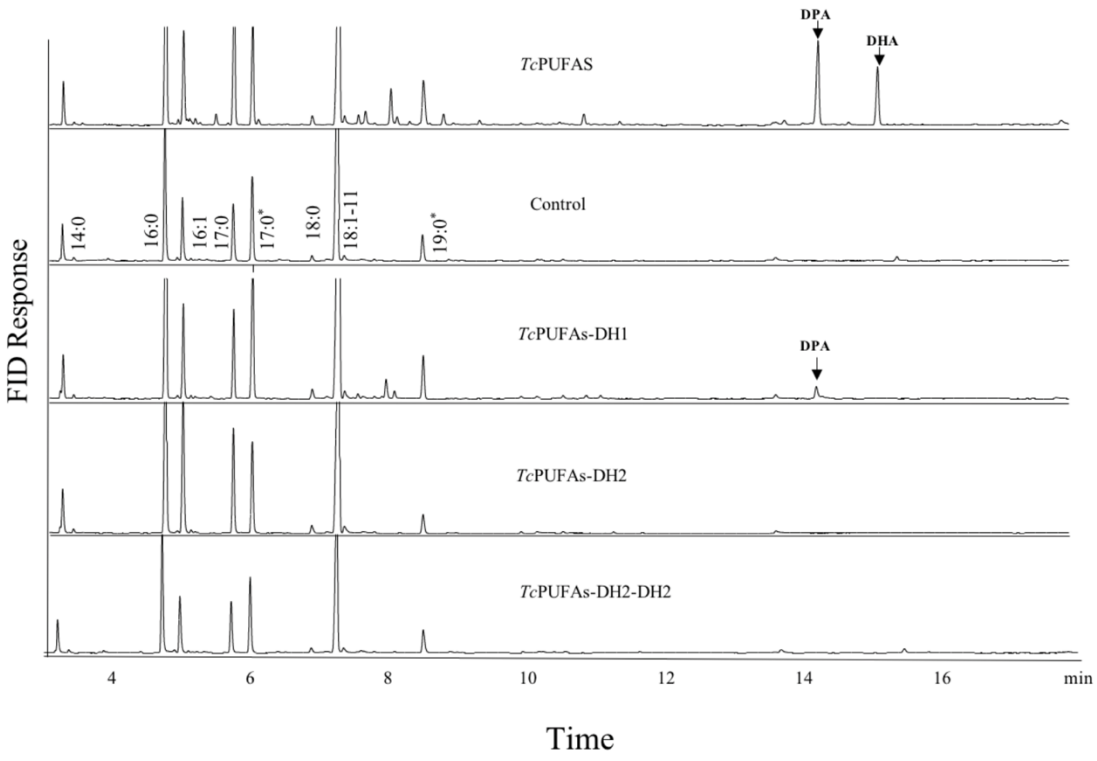

Figure 4.3.3 GC analysis of *E. coli* transformants expressing the PUFA synthase with deleted DH domains. TcPUFAS: *E.coli* expressing wild type PUFA synthase; Control, *E.coli* expressing a empty vector; TcPUFAs-DH1, *E.coli* expressing a PUFA synthase with a single DH1 domain; TcPUFAs-DH2, *E.coli* expressing a PUFA synthase with a single DH2 domain; TcPUFAs-DH2-DH2, *E.coli* expressing a PUFA synthase with two DH2 domains.

Table 4.3 Fatty acid compositions of *E. coli* transformants expressing the PUFA synthase with site-mutagenized and deleted DH2 domain. Values are the mean of three biological replicates with standard deviation.

	14:0	**16:0**	**16:1-9**	**17:0***	**18:0**	**18:1-11**	**19:0***	**22:5 (DPA-n6)**	**22:6 (DHA)**
TcPUFAS	11.53±0.18	65.68±1.96	21.93±0.56	26.18±0.72	2.38±0.31	78.25±2.91	10.35±0.64	11.40±2.08	5.49±0.88
TcPUFAS-DH1	10.55±0.25	59.55±1.31	22.31±0.44	22.30±1.15	2.23±0.21	60.27±3.68	5.98±0.57	0.25±0.03	
TcPUFAs-DH1-DH2*	11.85±0.39	64.00±3.00	29.14±1.74	20.21±1.43	2.01±0.25	68.31±3.71	4.35±0.64	0.51±0.04	

4.4 Expression and purification of the PUFA synthase for structural analysis

To express the PUFA synthase for protein purification, a *E. coli* high expression system was used to express three ORFs encoding three subunits of the PUFA synthase, each with a His-tag, in an operon under a T7 promoter (pET28a-ORF-ABC) in BL21 (DE3) (Meesapyodsuk & Qiu, 2016). The transformant was initially grown in LB broth at 37°C, and the protein expression was then induced with 0.5 mM IPTG at 16℃ for 36 hours. After induction, cells were harvested by centrifugation, and then disrupted by bead-beating. The lysates were centrifuged to separate the soluble fraction from solid cell debris. The PUFA synthase subunits in the supernatant were tentatively purified using a His-tag protein purification system. As shown in Figure 4.3.1, three protein bands in a SDS-PAGE with the size of 295 kDa, 223 kDa and 164 kDa corresponding to subunit-A, subunit-B and subunit-C of the PUFA synthase were observed in cell lysates from the transformant with pET28a-ORF-ABC, but not in cell lysates from the transformant with empty vector pET28a. All three proteins appeared in the supernatant fraction, although the amounts of subunit-C and subunit-B were much higher than that of subunit-A. This result indicates that three subunits of the PUFA synthase were all successfully, albeit unequally, expressed as soluble forms.

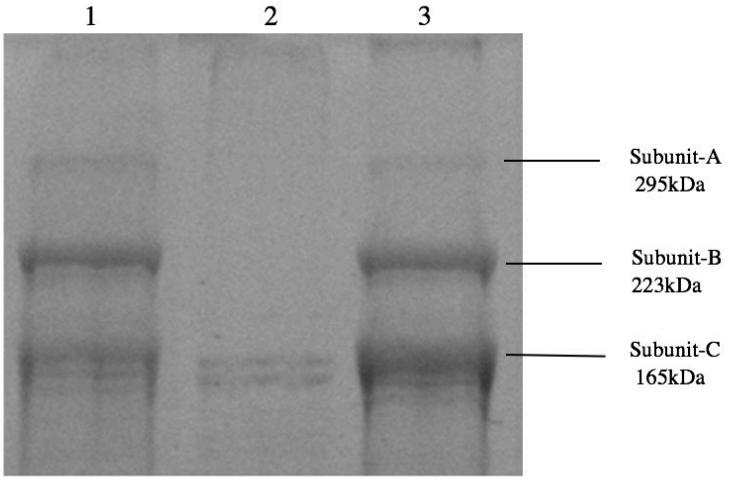

Figure 4.4.1 SDS-PAGE of protein profiles of E. coli BL21 (DE3) expressing the PUFA synthase. Lane 1, cell lysates from induced BL21 with pET28-ORF-ABC; Lane 2: cell lysates from induced BL21 with empty pET28a vector; Lane 3, supernatant of cell lysates from induced BL21 with pET28-ORF-ABC.

To obtain the optimal level of protein expression, various factors/conditions were evaluated in expressing pET28a-ORF-ABC, including in three different *E. coli* strains (BL21(DE3), Rosetta 2 (DE3) pLysS and Shuffle), with two different concentration of induction agent IPTG (0.5 mM and 1.0 mM), three different media (LB, LB with 10% glycerol and 2×YT with 10% glycerol), at three different temperatures (22℃, 30℃ and 37℃) and for four different induction times (12, 24, 36 and 48h). The results showed that BL21 (DE3) was the best host to express the plasmid (Figure 4.3.2). In addition, the 2×YT medium enriched with 10% glycerol was more effective than LB and LB with 10% glycerol in expressing the PUFA synthase. Induction with 1 mM IPTG gave a higher level of expression than that with 0.5 mM IPTG. No difference was observed in the expression among three different growth temperatures. However, the best expression result was obtained when the BL21 (DE3) transformant was grown in the 2×YT/10% glycerol with 1 mM of IPTG at 16℃ for 12 hours.

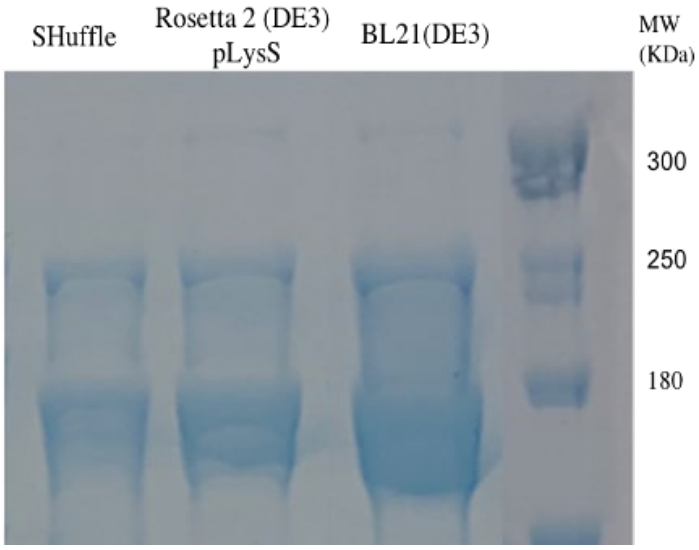

Figure 4.4.2 Effect of host strains on the protein expression of the PUFA synthase in *E. coli.*

Purification of the PUFA synthase subunits was also attempted using a His-tag protein purification system as each subunit of the PUFA synthase contains the N-terminal fusion of a 6-histidine tag in the expression construct pET28a-ORF-ABC, pET28a-ORF-C, pET28a-ORF-A. For doing this, the Ni-NTA resins were added to the supernatants of cell lysates from expression strains. The bound proteins were eluted from the resins by an elution buffer with imidazole. The

result showed that the expression of subunit-A was much higher in a single ORF construct (pET28a-ORF-A) than that in three ORF construct (pET28a-OFR-ABC), and all three subunits of the PUFA synthase could be considerably purified using Ni-NTA resins (Figure 4.4.3).

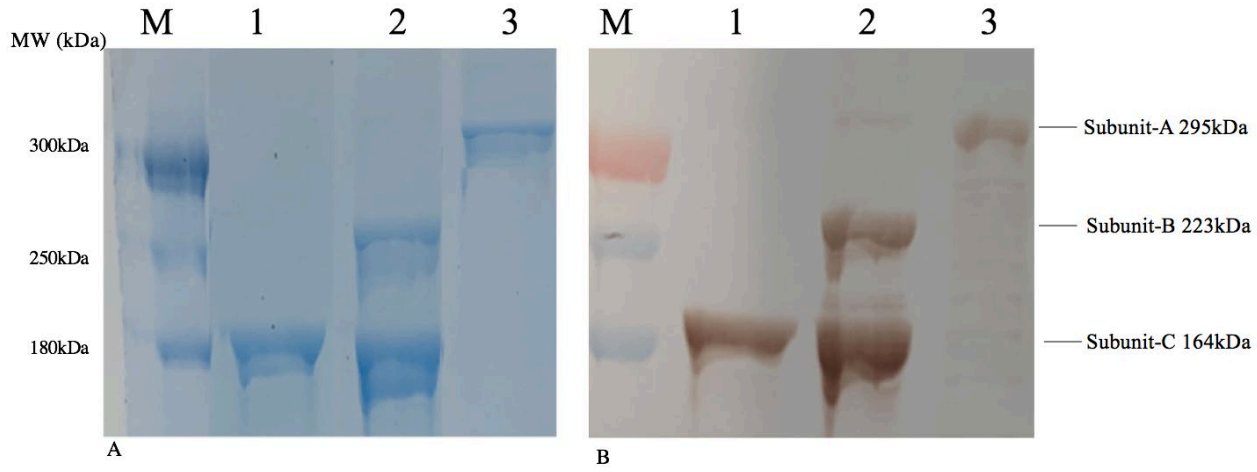

Figure 4.4.3 His-tag purification of the PUFA synthase subunits. A: SDS-PAGE analysis of purified protein samples. B: Western blot analysis of purified protein samples with anti-His tag HRP antibody. M, protein marker; Lane 1, purified protein from BL21 (DE3) expressing pET28a-ORF-C; Lane 2, purified protein from BL21 (DE3) expressing pET28a-ORF-ABC; Lane 3, purified protein from BL21 (DE3) expressing pET-28a-ORF-A.

5. DISCUSSION

Thraustochytrium sp. ATCC 26185 is a marine protist that is known for producing a high amount of VLCPUFAs such as DHA and DPA. Biosynthesis of these VLCPUFAs in the protist is solely catalyzed by a PUFA synthase comprising three large subunits, each with several catalytic domains (Meesapyodsuk and Qiu, 2016). However, the exact molecular mechanism of the PUFA synthase for positioning multiple double bonds in the VLCPUFAs remains unclear. Fatty acid synthesis generally proceeds with reiterative cycles using malonate as a two-carbon donor and acyl carrier protein (ACP) as covalent attachment for acyl chain extension. A full cycle of fatty acid biosynthesis is comprised of four reactions: condensation catalyzed by ketoacyl-ACP synthase (KS) for two carbon chain extension, reduction of a keto group by ketoacyl-ACP reductase (KR), dehydration by dehydratase (DH) for introducing a double bond, and reduction of the double bond by enoyl-ACP reductase (ER) (Qiu, 2003). Biosynthesis of VLCPUFAs catalyzed by a PUFA synthase is unique in that the process can periodically skip the enoyl reduction step of a full cycle, resulting in a double bond introduced by dehydratase being retained in an acyl chain. Therefore, positioning double bonds in the biosynthesis of VLCPUFAs must be a coordinate action of dehydratase and enoyl reductase or ketoacyl synthase.

A PUFA synthase in microorganisms possesses two types of dehydratase domains, polyketide synthase (PKS)-like dehydratase (DH_{PKS}) domain and FabA-like dehydratase (DH_{FabA}) domain (Xie et al., 2017). These two types of DH domains are required to introduce either trans or cis double bonds during the biosynthesis of VLCPUFAs. Probably, a trans double bond would be reduced by subsequent enoyl reductase activity, resulting in a saturated acyl chain, while a cis double bond in an acyl chain would be retained by ketoacyl synthase activity using an enoyl-ACP for subsequent condensation. A DH domain in Subunit-A of the PUFA synthase in *Thraustochytrium* is more similar to a polyketide synthase-type dehydratase domain; thus, it is likely for introducing a trans double bond. Two DH domains in Subunit-C, on the other hand, are more similar to FabA in *E. coli*, a bifunctional enzyme that catalyzes a dehydration of 3-hydroxydecanoyl-ACP to give trans-2-decenoyl-ACP, and then isomerizes trans-2-decenoyl-ACP into cis-3-decenoyl-ACP, an essential step for introducing a cis double bond in an acyl chain (Feng & Cronan, 2009).

To functionally characterize DH domains of the PUFA synthase for introducing multiple cis double bonds in VLCPUFAs, our focus was thus on the two FabA-like DH domains encoded by ORF-C of the PUFA synthase. Previously, Dairi and colleagues demonstrated that two adjacent

FabA-like DH domains of PUFA synthases from *Photobacterium profundum* and *Aureispira marina* were involved in positioning cis double bonds in ARA and EPA, respectively (Hayashi et al., 2019). However, functions of individual FabA-like DH domains in PUFA synthases from microorganisms remains undetermined. Our recent work showed that individual FabA-like DH domains from Subunit-C (DH1 and DH2) could functionally complement an *E. coli FabA* mutant, and overexpression of each DH domain in *E. coli* increased the fatty acid production (Xie et al., 2018). This indicates that a single FabA-like DH domain can functionally replace FabA and work together with other catalytic components of Type II fatty acid synthase in *E. coli* for the biosynthesis of unsaturated fatty acids. In this study, functions of two FabA-like domains from the PUFA synthase in *Thraustochytrium* was further analyzed by site-directed mutagenesis and domain deletion. If DH1 and DH2 domain are redundant in function, the mutation of catalytic residues in DH1 or DH2 should not have significant effect in VLCPUFA production. However, when the catalytic residue histidine in DH1 domain was changed to alanine, the PUFA synthase could not produce any VLCPUFAs, indicating the essentiality of the DH1 domain in the biosynthesis of VLCPUFAs. In contrast, when the histidine residue of DH2 was changed to alanine, the PUFA synthase could produce a small amount of DPA, but not DHA. This result was subsequently confirmed by individual DH domain deletion analysis. A single DH2 domain or double DH2 domains in the PUFA synthase did not produce any VLCPUFAs while a single DH1 domain could produce a small amount of DPA. These results provide unambiguous evidence that DH1 domain, but not DH2 domain of the PUFA synthase, is essential for the biosynthesis of VLCPUFAs; however, DH2 domain is required for the synthesis of DHA probably by introducing an omega-3 double bond in DPA.

In this study, for the first time, demonstrates that two FabA-like DH domains in microbial PUFA synthases do not function equally, each has distinct activity in the biosynthesis of VLCPUFAs. Although DH1 domain is more important than DH2 domain of PUFA synthases in the biosynthesis of VLCPUFAs, two DH domains cannot replace each other. Distinct nature of the two DH domains might lie in different dehydration activity towards acyl-ACP substrates with specific chain lengths. It has been shown that DH_{PKS} is important for the first dehydration process converting 3-hydroxybutyryl-ACP into 2-trans-butenoyl-ACP, which is subsequently reduced by enoyl reduction to give a saturated butyryl-ACP (Hayashi et al., 2019). A branching point of positioning ω-3 and ω-6 double bonds is whether hydroxyhexanoyl-ACP is utilized by DH_{PKS} or

$DH_{FabA,}$ which will result in different products (ARA or EPA) produced in *Photobacterium profundum* and *Aureispira marina*. However, the PUFA synthase in *Thraustochytrium* sp. ATCC 26185 synthesizes both ω-3 and ω-6 VLCPUFAs, which implies that two types of DH domains in the PUFA synthase might have promiscuous or overlapping substrates at the branching point. The fact that site-mutagenized or deleted DH1 domain resulted in the complete loss of activity and site-mutagenized or deleted DH2 domain maintained some activity in the biosynthesis of DPA might suggest that DH1 has lower affinity than DH2 to hydroxyhexanoyl-ACP for introducing the first double bond, giving an omega-3 VLCPUFA.

A previous complementation study showed that both DH1 and DH2 domains could functionally replace FabA for the biosynthesis of unsaturated fatty acids in an *E. coli* $FabA^{Ts}$ mutant (Xie et al., 2018). However, it was also evident that DH1 domain possessed significantly highly activity in the synthesis of saturated and unsaturated fatty acids than DH2 domain when expressed in both mutant and wild type strains (Xie et al., 2018). This study confirms that DH1 domain is more important than DH2 domain for the biosynthesis of VLCPUFAs, as mutation and deletion of DH1domain, other than DH2 domain, result in the complete loss of VLCPUFA-biosynthetic activity. Mutation and deletion of DH2 domains could still produce DPA, albeit at a small amount. However, it remains to be determined what structural factor is responsible for the differentation.

This research has also attempted the optimization of the PUFA synthase expression in *E. coli* and purification of the proteins for future structure analysis. To obtain a sufficient amount of purified proteins for structural study, several approaches have been used to optimize the expression of the PUFA synthase. Firstly, three different *E. coli* host strains (BL21(DE3), Rosetta 2 (DE3) pLysS, and Shuffle) were used to express the PUFA synthase construct pET28a-ORF-ABC. The three ORFs of the PUFA synthase are under a T7 promoter in the plasmid, the BL21 (DE3) strain that can produce T7 RNA polymerase is thus the first pick as a host for expressing the proteins (Pan & Malcolm, 2000). One of the most common problems encountered in protein expression is rare codons used in a heterologous gene. This is normally addressed by codon optimization of foreign genes prior to expression. However, due to the large size of three ORFs of the PUFA synthase (more than 20 kb), it is impractical to replace all the rare codons with common ones used in the host. Rosetta (DE3) pLysS is a BL21 derivative designed to enhance the expression of heterologous genes that contain codons rarely used in *E. coli* (Kim et al., 1998). Therefore, this

strain was also be selected as a host to express the PUFA synthase construct. Another problem in the expression of eukaryotic proteins in *E. coli* is disulfide bond formation and appropriate protein folding. Shuffle is an engineered *E. coli* strain that can catalyze the formation of disulfide bonds with enhanced capability of appropriate protein folding (Lobstein et al., 2012; Robinson et al., 2015). Therefore, this strain was also exploited to express the PUFA synthase. Among the three host strains, BL21 (DE3) proves to be the best for expressing the PUFA synthase. Next, three different media (LB, LB with 10% glycerol, and 2×YT with 10% glycerol) were attempted to grow the BL21 (DE3) transformants as culture medium composition can affect the recombinant protein expression by altering the bacterial host metabolism (Sivashanmugam et al., 2009). The 2×YT with 10% glycerol provides the best result probably due to a higher concentration of yeast extract in the medium with additional glycerol for increased stability of expressed proteins (Vagenende, Yap, & Trout, 2009). Moreover, two different inducer concentrations (0.5 mM and 1 mM), three growth temperatures (22℃, 30℃ and 37℃) and four different induction times (12, 24, 36 and 48 h) were also evaluated for yielding recombinant proteins. The best result is obtained on the condition with 1 mM IPTG induction at 16℃ for 12 hours. The expressed PUFA synthase subunits can be purified to certain extent using a His-tagged protein purification system. These results have laid the groundwork for future expression and purification of the PUFA synthase for the structure analysis.

In summary, our work from this study emphasizes a notion that two FabA-like DH domains (DH1 and DH2) of the PUFA synthase in *Thraustochytrium* function like FabA-type dehydratase to introduce cis double bonds in VLCPUFAs; however, each of them possesses distinct function and cannot replace one another. DH1 domain is essential for the biosynthesis of VLCPUFAs and DH2 is required for introducing an omega-3 double bond. The PUFA synthase must have both for the efficient production of VLCPUFAs. The preliminary results on the expression and purification of the PUFA synthase provide a step forward to our future work on the structure analysis of this enzyme.

6. CONCLUSION

Thraustochytrium sp. ATCC 26185 is a unicellular marine protist that can produce a high level of VLCPUFAs in membrane and storage lipids, using a PUFA synthase. Our previous studies indicate the coexistence of an aerobic pathway and an aerobic pathway in the protist, but the PUFA synthase in anaerobic pathway is solely responsible for the biosynthesis of VLCPUFAs (Meesapyodsuk & Qiu, 2016; Zhao et al., 2016). The PUFA synthase from *Thraustochytrium* comprises three subunits, subunit-A, -B and -C, each subunit comprises multiple catalytic domains. Three dehydratase (DH) domains have been identified in the PUFA synthase. One DH domain is located in subunit-A (DH-A) and the other two DH domains (DH1 and DH2) are located in subunit-C. The DH in subunit-A is more similar to polyketide synthase dehydratase domains, while the two DH domains in subunit-C are more similar to FabA in *E. coli*. *Thraustochytrium* sp. 26185 is a unicellular marine protist that can produce a high level of VLCPUFAs in membrane and storage lipids, using a PUFA synthase. Our previous studies indicate the coexistence of an aerobic pathway and an aerobic pathway in the protist, but the PUFA synthase in anaerobic pathway is solely responsible for the biosynthesis of VLCPUFAs (Meesapyodsuk & Qiu, 2016; Zhao et al., 2016). The PUFA synthase from *Thraustochytrium* comprises three subunits, subunit-A, -B and -C, each subunit comprises multiple catalytic domains. Three dehydratase (DH) domains have been identified in the PUFA synthase. One DH domain is located in subunit-A (DH-A) and the other two DH domains (DH1 and DH2) are located in subunit-C. The DH in subunit-A is more similar to polyketide synthase dehydratase domains, while the two DH domains in subunit-C are more similar to FabA in *E. coli*.

In this research, we aimed at functionally analyzing the function of two FabA-like DH domains in subunit-C of the PUFA synthase by mutagenesis and domain deletion. On the basis of the sequence analysis including multiple sequence alignment and homology model, several key residues probably important for catalysis were identified in DH1 and DH2 of ORF-C for mutagenesis. When the catalytic residue histidine in DH1 domain was changed to alanine, the PUFA synthase could not produce any VLCPUFAs; while when the histidine residue of DH2 was changed to alanine, the PUFA synthase could produce a small amount of DPA, but not DHA. Western blot analysis of PUFA synthases with individually mutated residues showed that they were successfully expressed as soluble forms. The site-mutagenetic results were further confirmed by individual DH domain deletion, where a single DH2 domain or double DH2 domains in the PUFA synthase did

not produce any VLCPUFAs, but a single DH1 domain could produce a small amount of DPA. These results clearly indicate that DH1 domain is essential for the biosynthesis of VLCPUFAs while DH2 domain is required for the synthesis of DHA. Furthermore, expression and purification of the PUFA synthase in *E. coli* were also attempted in this study. The best result was obtained when the BL21 (DE3) transformant expressing the PUFA synthase was grown in the 2×YT/10% glycerol with 1 mM of IPTG at 16℃ for 12 hours.

7. FUTURE DIRECTION

VLCPUFAs are essential for humans and animals. The demand for these fatty acids such as DHA and EPA is growing due to the increased awareness of their health benefits on the protection against cardiovascular diseases and enhanced performance of our eyes and brains (Berman et al., 2010; Hibbeln et al., 2007; Makrides et al., 2010). The traditional source of VLCPUFAs for dietary supplementation is oceanic fish. However, with the issues of over-fishing and ocean pollution, sustainability of this source is questionable. Therefore, the source for VLCPUFAs alternative to traditional fish oil is needed to meet the increasing demand. Microorganisms such as oceanic bacteria, microalgae and protists can *de novo* synthesize VLCPUFAs. Some of these species can accumulate a large amount of DHA when appropriate growth conditions are in place. Thus, they are potential suppliers of these fatty acids to meet the market demands. However, growing VLCPUFA-producing microbes and extracting oil from them can be challenging and costly. In this situation, production of these fatty acids in transgenic oilseed plants or oleaginous microbes by genetic engineering is another attractive alternative. For this, mechanistic studies like this one on functional analysis of DH domains of a PUFA synthase in the VLCPUFA biosynthesis is critical in implementing the transgenic production of these fatty acids in heterologous systems.

In nature, biosynthesis of VLCPUFAs goes through two different pathways. The aerobic pathway involves aerobic desaturations and elongations, while the anaerobic pathway is catalyzed by a single mega-enzyme, PUFA synthase. The anaerobic pathway is more efficient in producing VLCPUFAs since it has less catalytic steps involved with fewer intermediates produced. The PUFA synthase pathway has been identified in many microorganisms where it is responsible for the synthesis of various VLCPUFAs. Recently, this pathway has also been successfully reconstituted in heterologous systems such as *E. coli* and *Lactococcus lactis* to produce EPA or/and DHA (Amiri-Jami et al., 2014; Meesapyodsuk & Qiu, 2016; Metz et al., 2001). However, the molecular mechanism of how multiple catalytic domains in a PUFA synthase coordinate with each other to carry out the VLCPUFA synthesis remains unclear. Therefore, understanding the catalytic mechanism would help improve the production of VLCPUFAs in native producers and optimize the transgenic production of these fatty acids in heterologous systems. In addition, PUFA synthases from bacteria, microalgae and protists produce different VLCPUFA profiles. For instance, PUFA synthase from bacterial *Aureispira marina* and *Moritella marina* synthesize ARA and DHA,

respectively; while PUFA synthases from *Schizochytrium* and *Thraustochytrium* produce both ω-3 and ω-6 VLCPUFAs. Therefore, understanding the functions of individual domains in these PUFA synthases would offer strategy to design super PUFA synthases with specific fatty acid profiles by domain swapping and domain deletion among PUFA synthases.

Three-dimensional structure information of proteins is fundamental for us to understand the catalytic mechanism of enzymes. Therefore, another future direction can be the elucidation of the structure of the PUFA synthase. PUFA synthase is a mega-enzyme comprising three large subunits made of a total of more than 7000 amino acids. Therefore, it is challenging to obtain a sufficient amount of proteins with enough purity and quality for three-dimensional structure analysis. Our work in this study have laid a groundwork for future expression and purification of the PUFA synthase for the structure analysis.

Notes: Some of the thesis results are published as part of an article entitled as "**Distinct Functions of two FabA-like Dehydratase Domains of Polyunsaturated Fatty Acid Synthase in the Biosynthesis of Very Long Chain Polyunsaturated Fatty Acids"** in Environmental Microbiology, 2020.

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